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A SERIES OF FULLY SUBSTITUTED ETHYLENEDIAMINES

BY

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(RECEIVED FEBRUARY 18, 1955)

N-dimethylaminoethyl-*N*-benzylaniline (2339 RP or "Antergan") has long been known as a histamine antagonist (Halpern, 1942). It is five to fifteen times more potent than its *N*-ethylaniline analogue (2325 RP).

Nickerson and Gump (1949) demonstrated a more potent antiadrenaline activity in certain compounds after increasing the size of one aryl residue. Accordingly a series of analogues of 2339 RP and 2325 RP was synthesized (Chapman, James, and Williams, 1952) in which the size of one aryl nucleus was increased by replacing the phenyl by a naphthyl group. The ethylenediamine side chain can be introduced in the 1- or 2-positions of this nucleus (see Table I). A brief report of certain activities has been made (Tonks, 1953). The neuromuscular blocking activity of these substances, which resembles that of decamethonium, has been described by Graham and Tonks (1954). We now describe the antihistamine, antiadrenaline, local anaesthetic, and other properties of these compounds. For convenience they are labelled T1 to T8.

METHODS

Comparison was made with 2339 RP and 2325 RP throughout the tests. The naphthyl analogues (T1–8) of the RP compounds are oily bases. They were dissolved in acetic acid (33%) and the volume was adjusted with citric acid/phosphate buffer of *p*H 6.5.

Antihistamine activity was investigated on the response of the blood pressure of atropinized and anaesthetized cats, dogs, and rabbits injected intravenously with histamine (1–4 μg./kg.) before and 5 min. after graded doses of T1–T8. The mode of action was studied by the method of Chen and Russell (1950), together with the course of activity with time. Guinea-pigs of about 300 g. were exposed individually to an aerosol of histamine solution (0.5%) and the time taken to collapse from asphyxia noted. The animals were resuscitated and used in groups of 10 at intervals of 7 days, when graded doses of T1–T8 were injected subcutaneously 1 hr. before exposure. The effect of T1–T8 on the vascular reactions of guinea-pig skin was investigated by the method of Miles and Miles (1952). In addition their effect on smooth muscle (guinea-pig ileum, rat uterus, perfused vessels of rabbit ear) and cardiac muscle (perfused cat and rabbit heart) was examined. Gastric juice was collected from 8 cats under chloralose by cannulation of the pylorus after ligation of the oesophagus and wash out. Histamine (15 μg./0.5 ml./min.) was infused intravenously for 45 min. and the gastric juice collected until flow ceased. One hour later T1 (10 mg./kg.) was injected intravenously and after 15 min. the histamine infusion was repeated. One hour after flow ceased the infusion was repeated for the third time. T1 was omitted in 3 cats. Histaminase was prepared from fresh hog-kidney and its activity on the substrate was tested alone and in the presence of T1 and 2339 RP (2×10^{-3} to 7×10^{-3}) according to Kapeller-Adler (1951). Antiadrenaline activity was examined on the blood pressure responses of atropinized spinal cats, cats anaesthetized with chloralose, rabbits anaesthetized with urethane, and dogs anaesthetized with sodium pentobarbitone or with sodium thiopentone followed by chlorbutol (1 ml. 40% w/v soln. in 70% ethanol/kg. intraperitoneally). The technique of Chen, Nash, and Russell (1950) was applied, and the duration of action of effective doses noted. The effect of T7 (7.5 mg./kg. intraperitoneally) on the hyperglycaemic response to adrenaline (40 μg./kg. subcutaneously) was observed in 6 rabbits. These were separately caged for 24 hr. without food before individual fasting blood-sugar levels were determined by the modified Folin-Malmros method (Landgrebe and Munday, 1954). They were injected subcutaneously at 7 day intervals with 0.5 ml. saline, adrenaline, T7, or T7 followed 30 min. later by adrenaline, and hourly individual blood-sugar levels were estimated for 5 hr. The effect of the compounds on the responses of smooth muscle (gut, pregnant and non-pregnant uterus of cat and rabbit, perfused vessels of rabbit ear) and cardiac muscle (cat and rabbit heart, rabbit auricles) to adrenaline was also tested. Homogenized guinea-pig liver was used as a source of amine oxidase and the action of the compounds on this enzyme was examined manometrically with adrenaline as substrate. Lineweaver and Burk's (1934) method was used to investigate the type of inhibition exerted. The concentrations of adrenaline substrate were 0.025 M to 0.001 M and those of the inhibitors 2×10^{-4}, 10^{-4}, and 2×10^{-5}.

Local Anaesthesia.—The ethylenediamines were compared with procaine, with and without adrenaline, and with cocaine as infiltration anaesthetics by Bülbring and Wajda's method (1945). Duration of surface anaesthetic activity was measured on rabbit corneas (6 rabbits/compound) with the aid of a light von Frey bristle. The solution under test was kept in contact with the cornea for 1 min. The eye was then flushed with saline and touched thereafter four times every 2 min. until the corneal reflex reappeared. The right eyes of 3 rabbits and the left eyes of another 3 rabbits were used for each compound. After recovery, cocaine was tested in the unused eyes. Three concentrations of each compound were tested at intervals of 7 days, the highest used for T1–8 being 0.125 to 0.5% w/v; for cocaine 2% w/v, and for 2339 RP and 2325 RP 10% w/v.

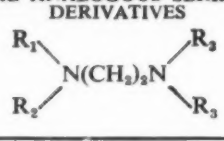
Results

Toxicity

Table I shows the structures of the compounds and the toxicity of 2339 RP, 2325 RP and their naphthyl analogues estimated in mice and rats by Karber's method. The initial effects of a lethal dose of any of the compounds injected intraperitoneally are observed within 5 min. and death occurs within 45 min., whereas it occurs within 12 to 24 hr. after subcutaneous injection. Stimulation of the central nervous system is observed in all animals. Tremor and hyperpnoea may be followed by tail stiffening, clonic convulsions and terminal asphyxia. The stimulant action is most pronounced in the *N*-ethyl analogues; mice surviving the acute toxicity test convulse for 3 to 4 hr. The RP compounds are less active in this respect. The growth of litter-mate groups of rats is unaffected by the compounds in maximal tolerated doses (T1 20 mg./kg., T7 10 mg./kg. subcutaneously, or 40 mg./kg. of either orally) and blood tests after 6–7 weeks' administration reveal no abnormality.

Table I

THE STRUCTURE AND TOXICITIES (MG./KG.) OF 2339 RP, 2325 RP, AND THE ANALOGOUS SERIES OF NAPHTHYL DERIVATIVES



Code No.	R_1	R_2	R_3	LD50 Mice i.p.	LD50 Mice s.c.	Min. LD i.p. Mice	Min. LD i.p. Rats
2339 RP (antergan)	Benzyl	Phenyl	Methyl	175	400	110	120
2325 RP	Ethyl			500	1150	350	350
T1	Benzyl	1-Naphthyl	Methyl	135	463	75	90
T2	Benzyl	1-Naphthyl	Ethyl	63	300	35	45
T3	Benzyl	2-Naphthyl	Methyl	265	740	180	135
T4	Benzyl	2-Naphthyl	Ethyl	128	459	100	90
T5	Ethyl	1-Naphthyl	Methyl	121	443	80	80
T6	Ethyl	1-Naphthyl	Ethyl	65	306	40	45
T7	Ethyl	2-Naphthyl	Methyl	53	287	32	50
T8	Ethyl	2-Naphthyl	Ethyl	35	172	25	40

All the compounds when injected intradermally in high concentrations (0.5% (w/v)) produce an area of inflammation which ulcerates in 48 hr. Microscopically the ulcers show a more or less complete destruction of the epidermal architecture with infiltration of the dermis by polymorphs in dogs and by eosinophils in rabbits. The relative potencies of the compounds in producing ulceration in the skin of guinea-pigs at 48 hr. were measured by determining minimal effective concentrations. The 1-naphthyl compounds are less effective than their 2-naphthyl isomerides in the benzyl series, while the converse is true for the ethyl series. The influence of the side-chain position on general toxicity is the reverse of that observed for tissue damage.

Antagonism to Histamine, Adrenaline, etc.

Antihistamine activity is slight in the naphthyl derivatives except for T1, which is the most active member of the series. The results are given in Table II. The antihistamine activity is specific, whereas this is not so with 2339 RP and 2325 RP, in which an atropine-like activity is present. The onset of antihistamine activity is rapid—within 5 min.—and the duration of activity of T1, 10 mg./kg., injected intravenously in dogs is 2.5 hours, whereas T7, 20 mg./kg., is effective for one hour. Application of Gaddum's equation (1943) to the

Table II

THE ED50 OF THE ETHYLENEDIAMINES AS INHIBITORS OF (1) THE DEPRESSOR ACTION OF HISTAMINE (2 μG./KG.) IN THE CAT, (2) ASPHYXIATION OF GUINEA-PIGS WITH HISTAMINE AEROSOL, (3) CONSTRICTION OF PERFUSED RABBIT EAR VESSELS BY 0·5 μG. HISTAMINE, AND (4) THE CONTRACTION OF ISOLATED GUINEA-PIG ILEUM TO HISTAMINE 10^{-8}

Compound	(1) Blood Pressure (mg./kg., i.v.)	(2) Bronchi (mg./kg. s.c.)	(3) Ear Vessels (μg.)	(4) Ileum (μg./100 ml.)
N-benzyl series:				
2339 RP	1·4	0·6	0·0125	0·065
T1	3·3	5·6	0·280	3·4
T2	17·0	0*	—	4·0
T3	20·0	0*	—	5·0
T4	24·0	0*	—	16·0
N-ethyl series:				
2325 RP	8·0	9·8	0·16	2·5
T5	11·0	0*	2·0	20·0
T6	13·0	0*	—	24·0
T7	15·0	0*	—	31·0
T8	18·0	0*	—	51·0

* Less than 50% effect after maximal tolerated doses.

determination of equi-effective amounts of histamine in the presence of increasing amounts of T1 indicates that the antagonism is competitive at all doses.

Only compound T1 displays notable activity in protecting guinea-pigs exposed to histamine spray. Maximum tolerated doses of the remaining naphthyl derivatives (16 mg./kg. subcutaneously) prolong the time to asphyxiation by 15–28%, whereas 2339 RP gives a figure of 100%, T1 81% and 2325 RP 64% at this dose.

The results of the assays on isolated guinea-pig ileum are shown in Table II. T1 is 30 times more effective in antagonizing the response to histamine than the response to ACh and barium, while T2–8 are only ten times more effective in this respect. 2339 RP shows atropine-like activity in addition to being a more potent antihistamine. The inhibition of the response of the perfused vessels of the isolated rabbit ear to histamine by T1 and its *N*-ethyl analogue T5 is also shown in Table II.

From the ED50's, 2339 RP is seven times more active than T1 in inhibiting the flare produced in guinea-pigs by intradermal histamine. The volume of gastric juice in cats after intravenous histamine is increased fourfold by a dose of T1 (10 mg./kg.) which completely inhibits the vasodepressor action of histamine. The acidity is approximately the same as for the histamine controls. The stimulant action of histamine on isolated perfused hearts is readily abolished by these compounds; thus the ED50 for this action on the Langendorff preparation is 6 μg. for 2339 RP, 9 μg. T1, 12.5 μg. 2325 RP and 21 μg. T5. As with all antihistamines so far tested the response of the rat uterus to histamine is unaffected.

. T1 is a weak inhibitor of histaminase, a concentration of 10^{-3} reducing activity by 40%. 2339 RP is inactive.

Antiadrenaline activity is weak in this series with the exception of T7, of which 2 mg./kg., i.v., reverses the pressor response to 4 μg./kg. adrenaline and 2 μg./kg. noradrenaline in the cat and dog. As a group the compounds may have one or more of three effects on the pressor response to adrenaline and noradrenaline (viz., potentiation, inhibition or reversal). T7 produces potentiation in the spinal cat only, T8 in spinal and anaesthetized cats only and the remaining compounds in the cat, dog, and rabbit. All except T2 inhibit the responses to adrenaline and noradrenaline in the dog, while 2325 RP and T7 also inhibit them in the cat. T7 produces reversal of the pressor responses in the cat and dog and a maximum tolerated dose of T4 (20 mg./kg.) does so in dogs. The potentiating effect is strongest in the compounds which have the weakest inhibitory action. The minimal effective dose of T7 for the reversal of adrenaline is the same in rabbit, cat, and dog (2 mg./kg.). As with the more potent 2-haloalkylamines (Graham and Lewis, 1954) the pressor response to injected adrenaline in the cat and dog is more easily inhibited than that to noradrenaline. Similarly, the contraction of the nictitating membrane of cats after injected adrenaline is more easily inhibited than that caused by injected noradrenaline or stimulation of the cervical sympathetic nerve. Responses to pre- and postganglionic stimulation are identical in the presence of T7. This compound does not exert ganglion blocking action in doses effective at the neuromuscular junction (Graham and Tonks, 1954). The pressor response to occlusion of the carotid arteries is inhibited to a lesser extent, as is the response to stimulation of the splanchnic nerve. The activities of SY28 (haloalkylamine) and T7 are compared in Table III.

TABLE III

ED50 OF SY28 AND T7 (MG./KG.) IN ANTAGONIZING EQUI-PRESSOR RESPONSES TO ADRENALINE AND NORADRENALINE, THE PRESSOR RESPONSE TO CAROTID OCCLUSION, AND THE CONTRACTION OF THE NICTITATING MEMBRANE TO ADRENALINE AND CERVICAL SYMPATHETIC STIMULATION, IN CATS AND DOGS

Preparation	Treatment	SY28	T7
Cat pressor response	Adrenaline	0·04	0·30
	Noradrenaline	0·10	0·90
Cat nictitating membrane	Adrenaline	0·10	0·70
	Stimulation	4·0	—
Dog pressor response	Adrenaline	0 04	0·30
	Noradrenaline	0·10	0·95
	Carotid reflex	0·60	20 0

In the rabbit, cat, and dog the antagonism of the responses to graded equi-pressor amounts of adrenaline and noradrenaline by T7 is competitive at all dose levels. The onset of antagonism with effective doses occurs within 5 min. Potency and duration go together; thus T7, 20 mg./kg., reverses the pressor response to adrenaline and abolishes that to noradrenaline in dogs. The antagonism lasts for 5 hr. and 3.5 hr. respectively. An identical dose of T3 (benzyl analogue of T7) produces a 50–60% inhibition of the pressor response to adrenaline which lasts for 3 hr. This compound displays very little antagonism to noradrenaline.

Compound T7 (7.5 mg./kg.) reduces the hyperglycaemic response of rabbits to subcutaneous injection of adrenaline by 40%. At peak blood sugar levels there was considerable scatter in individual readings, but a "t" test indicated a significant difference ($0.05>P>0.02$).

Non-toxic concentrations of the naphthyl derivatives do not affect the inhibitory action of adrenaline and noradrenaline on rabbit gut or non-pregnant cat uterus. The responses of isolated auricle or perfused heart are not affected. In contrast stimulation of rabbit uterus, pregnant cat uterus and perfused rabbit ear vessels by both amines is inhibited. In the perfused vessels, T7 is ten times more active than T3 in antagonizing adrenaline, but less active against histamine. The ratio of the ED50 as an antihistaminic to that as an antiadrenaline agent is 12.5 for T7 and 0.5 for T3 in this preparation.

All the compounds investigated inhibit the activity of amine oxidase on adrenaline *in vitro*. The ED50 for T2, the most active potentiator of the vasopressor action of adrenaline, is 10^{-4}, and for T7, the most active inhibitor, 2×10^{-4}. The nature of this action was examined according to Lineweaver and Burk (1934). The velocity constant for each of three concentrations of the compound under test (2×10^{-4}, 10^{-4}, 2×10^{-5}) was determined by multiplying the factor 2.303 by the slope of the curve relating log rate of uptake of oxygen to time for several concentrations (0.025M to 0.001M) of the substrate. The relation between the reciprocals of the velocity constants and the reciprocals of the substrate concentrations is linear, and the intercept of the ordinate axis (velocity constant) increases with the concentration of inhibitor, as does the slope. This illustrates the non-competitive nature of the antagonisms.

Local Anaesthesia

The potencies of these substances as infiltration anaesthetics determined in guinea-pigs relative to procaine are given in Table IV. All the naphthyl derivatives are more active than procaine and T1 is more potent than 2339 RP. Adrenaline increases the potency of procaine tenfold and that of T1 twofold. In view of the necrosis produced when these compounds are injected into the skin, the animals were sacrificed immediately after the test and histological examinations made. No oedema or other evidence of damage was detected with the concentrations used (maximum 0.5% w/v). The surface anaesthetic activity of the ethylenediamines was compared with cocaine, using the corneal reflex in the rabbit. The mean and standard error of 60 readings for each concentration of cocaine (with the range and standard errors of individual sets of readings in parentheses) determined in different groups of rabbits was as follows: $0.5\% = 13 \pm 1.1$ (12.0 ± 1.5 to 13.5 ± 3.4), $1.0\% = 28 \pm 0.98$ (21.5 ± 1.8 to 36.7 ± 4.0), $2.0\% = 43.2 \pm 0.96$ (34.8 ± 3.1 to 54.5 ± 1.2). The regression coefficients of the dose-response relations are almost identical for each compound, the response being the duration of anaesthesia. The potency figures are given in Table IV, column 3. The inactivity of 2325 RP compared with 2339 RP has been recorded by Halpern, Perrin and Dews (1947). No conjunctivitis results from the use of these drugs. Mydriatic activity was not detected in compounds T1–8.

TABLE IV

THE POTENCIES OF COCAINE AND PROCAINE, 2339 RP, 2325 RP, AND T1–8 AS LOCAL ANAESTHETICS DETERMINED BY INFILTRATION OF GUINEA-PIG SKIN AND SURFACE ACTIVITY (CORNEAL REFLEX IN RABBITS), AS INHIBITORS OF THE ACTION OF ACh ON FROG RECTUS, INHIBITORS OF THE PERISTALTIC REFLEX IN RABBIT GUT, AND AS ANTIHISTAMINES (CAT BLOOD PRESSURE)

Compound	Potencies				
	Anaesthetic		Anti-ACh	Anti-peristaltic	Anti-hist.
	Infiltr.	Surface			
Procaine ..	1·0	0	1·0	0·2	—
Procaine and adrenaline (10^{-5}) ..	10·0	0	—	—	—
Cocaine ..	6·6	1·0	5·0	1·0	—
2339 RP ..	2·0	0·95	1·25	1·4	1·0
2325 RP ..	0·6	0	0·33	0	0·18
T1	2·6	22	2·5	20	0·4
T1 and adrenaline (10^{-5})	5·2	—	—	—	—
T2	2·1	17	2·4	18	0·08
T3	2·1	19	2·0	18	0·07
T4	2·1	16	1·7	18	0·06
T5	1·2	3·3	1·25	5	0·13
T6	1·5	5·9	1·2	7	0·11
T7	1·4	4·8	1·25	5	0·10
T8	1·5	5·7	1·25	6	0·08

Application of a 5% solution of T1 for 1 min. to a localized area of the tibial nerve in cats anaesthetized with chloralose abolishes reflex contraction of the tibialis anticus muscle when suitable stimuli are applied distal to the block. The effect is apparent for at least 2 hr. after washing off the drug. A 20% solution has a similar effect on conduction in the motor nerve.

The potencies of the compounds relative to cocaine and procaine in abolishing the response of isolated frog rectus muscle to standard doses of ACh are recorded in Table IV, column 4, and potencies in abolishing the peristaltic reflex in isolated segments of rabbit gut (Feldberg and Linn, 1948) in column 5. Cocaine, procaine, and the ethylenediamines were in contact with the cornea, frog rectus, and rabbit gut for 1 min. The antihistamine potencies determined on the blood pressure of cats are given in column 6 for comparison.

The ethylenediamines stimulate the central nervous system at all levels. In low doses (0.0625 of the LD50 administered subcutaneously to mice)

T1 is less potent than leptazole as an analeptic, whereas T5 is twice as active. In higher doses both compounds are toxic. All the compounds in non-toxic doses produce respiratory stimulation in dogs anaesthetized with pentobarbitone sodium.

Discussion

Increasing the size of 2339 RP and 2325 RP by changing the phenyl nucleus to naphthyl increases the toxicity and the local anaesthetic potency. Replacement of dimethyl by diethyl in the sidechain of this series increases toxicity. This relation does not hold in the benzyl derivatives for local anaesthetic activity. Alteration of the sidechain from the 1-naphthyl position to the 2-naphthyl position increases toxicity and local anaesthetic activity in the ethyl series and decreases both in the benzyl series. By increasing the molecular weight of the aryl residue of 2339 and 2325 RP, antihistamine activity is reduced, 2339 RP being the most active of the series. It is apparent from this investigation that benzyl substitution on the aromatic amino group and dimethyl substitution on the aliphatic amino group give the greatest antihistamine activity. In addition the 1-naphthyl position of the sidechain is superior to the 2 position. *N'*-ethyl-*N'*-2-naphthyl-*N*:*N*-dimethyl ethylenediamine (T7) has much the highest antiadrenaline activity.

Muscular paralysis may be a factor in respiratory failure in cats and dogs following toxic doses of these compounds. The potentiation of pressor responses to adrenaline and noradrenaline is inversely related to their efficiency as antagonists. Thus T7, the active antiadrenaline member of the series, produces little potentiation, whereas the converse is true for T2, the least active. Similar instances were observed by Graham and Lewis (1953) in the related field of 2-haloethylamines. Only very high concentrations of T1–8 act directly on cardiac and smooth muscle cells to impair function and sensitivity. The antagonism to both adrenaline and histamine is peripheral and competitive, and, just as exogenous histamine is more readily inhibited than endogenous histamine, so exogenous or injected adrenaline and noradrenaline are more easily antagonized than are the transmitters released by stimulating adrenergic nerves. Both effects develop quickly, but the duration differs markedly. There is no obvious relation between molecular structure and antihistamine and antiadrenaline activity, the strongest antiadrenaline agent being a feeble, but not the weakest, antihistamine, and vice versa.

It has been suggested (Burn, 1950a and b) that a relation exists between antihistamine and local anaesthetic action on the one hand and anti-ACh action and local anaesthesia on the other. The substituted ethylenediamines T1–8, which conform to the formula aromatic residue (lipophilic)—intermediate chain—amino group (hydrophilic) suggested by Löfgren (1948) are active local anaesthetics, but no relation between potency in this respect and antihistamine activity has been found. Although T1 is the most active local anaesthetic its antihistamine potency is less than one-half that of 2339 RP.

The benzyl derivatives as a group are more potent infiltration anaesthetics and produce necrosis more easily than the phenyl compounds, but in half the members of the benzyl series the two properties do not increase together. Concentrations which are only one-eighth the strength required to produce cloudiness of the cornea cause anaesthesia of the surface for one hour. The measure of this activity and of the power to inhibit the peristaltic reflex in segments of gut are almost identical (see Table IV). The latter action is unlikely to be due to necrosis and it is concluded that anaesthesia is not secondary to gross tissue damage. Eerden (1948) suggested, as the result of experiments with 2339 RP, that anaesthesia of the cornea is connected with a reversible coagulation of intracellular proteins. The reversible opacity of the cornea with high concentrations of cocaine and T1–8 indicates that this factor may play a part in their activity. The parallelism between the activity of some infiltration anaesthetics and their power to antagonize the action of ACh on frog rectus muscle was first pointed out by de Elío (1948). It has been suggested that this is a causative relation. Such parallelism is also seen with the ethylenediamines between their potencies as intradermal local anaesthetics and as antagonists of ACh on frog rectus, although they also antagonize the responses to potassium chloride (Graham and Tonks, 1954). There is an appreciable increase in the anaesthetic potencies of the ethylenediamines relative to cocaine on the avascular cornea compared with their potencies intradermally. Cocaine produces vasoconstriction in skin, the ethylenediamines do not. Added adrenaline potentiates the activity of procaine but has less effect on T1–8 presumably because at the concentrations used they are active antagonists of adrenaline.

Summary

1. The properties of 8 compounds related to 2339 RP (Antergan) and 2325 RP have been examined. They are *N'*-benzyl (or ethyl)-*N'*-1-(or

2-) naphthyl-*N*:*N*-dimethyl-(or diethyl)-ethylene-diamines.

2. The LD50's in mice and minimal lethal doses in rats and mice are reported.

3. All the compounds exhibit competitive antihistamine activity which is less than that of 2339 RP. The benzyl-naphthyl-dimethyl derivative is the most active of the series.

4. The ethyl-2-naphthyl-dimethyl derivative is the most active antiadrenaline agent; the action is competitive. Activity is about 15% that of SY28. The effect of noradrenaline and sympathetic activity is less easily inhibited than that of adrenaline.

5. The compounds are more effective than procaine as infiltration anaesthetics and more potent than cocaine as surface anaesthetics.

6. There is no relation between local anaesthetic potency, antihistamine or antiadrenaline activity.

7. They are stimulants of the central nervous system.

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ACCELERATION OF THE ENZYMIC HYDROLYSIS OF BENZOYLCHOLINE

BY

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During routine examination of new compounds in these laboratories, one rarely finds substances which enhance the action of cholinesterases. One such substance, however, is (+)-1-*cyclo*hexyl-1-phenyl-3-pyrrolidinopropan-1-ol methiodide ((+)-procyclidine methiodide, (+)-tricyclamol iodide). Activation of cholinesterases has been claimed for histidine, and some other amino acids, by Aron, Herschberg, and Frommel (1944), and Frommel (1946, 1947); for vitamin E by Bloch (1942); for folic acid by Davis (1946a, b, c); and for dihydrohydroxycodeinone hydrochloride (Eukodal) by Hazard, Cornec, and Pignard (1950). Furthermore, Todrick, Fellowes, and Rutland (1951) showed that certain alcohols activated the hydrolysis of acetylcholine by rat brain and human erythrocytes; the activation was less marked when the specific substrates, benzoylcholine and acetyl-β-methylcholine, were used with the cholinesterases of horse serum and rat brain, respectively.

This paper is concerned with analogous effects of a number of commonly used drugs on certain cholinesterase systems.

METHODS

Cholinesterase activity was determined by the Warburg manometric method as previously described (Fraser, 1954). The drug and cholinesterase preparation were kept in contact for 30 min. before starting the main experiment. The rates of substrate hydrolysis by cholinesterase in the presence of each compound over a wide range of concentrations were compared with that in its absence. The results, given as percentage acceleration of the normal rate of hydrolysis, are positive for acceleration and negative for deceleration, or inhibition. The possibility of liberation of gas from the interaction of the compounds under test and the esterase preparation was eliminated by suitable controls. Atropinase activity was similarly determined in the Warburg respirometer, with atropine sulphate as substrate, at a final concentration of 0.002 M.

Cholinesterase Preparations.—The tests were usually performed with the cholinesterases of human blood (Fraser, 1954). Plasma was the source of pseudo-cholinesterase, which had an activity of 1,500 μl. CO_2/ml./hr. with benzoylcholine (0.015 M) as substrate; lysed red cells were the source of the true cholinesterase, which had an activity of 2,000 μl. CO_2/ml./hr. with acetyl-β-methylcholine (0.03 M) as substrate. Sometimes rabbit plasma or horse serum was used as a source of cholinesterase. Unless otherwise stated, the substrates and their final concentrations were as follows: acetylcholine chloride, 0.015 M; acetyl-β-methylcholine chloride, 0.03 M; benzoylcholine chloride, 0.015 M; butyrylcholine iodide, 0.015 M; tributyrin, 0.03 M; and succinyldicholine dichloride, 0.015 M.

RESULTS

Effect on Pseudo-cholinesterase

The Procyclidine Group.—The racemic and optically active forms of the hydrochloride, methiodide and ethiodide of procyclidine were studied. The nitrogen in the last two is quaternary, whereas in the hydrochloride it is tertiary. Duffin and Green (1955) have found that the *laevo* compounds possess a considerable degree of atropine-like activity. This is virtually absent from the *dextro* forms, and the racemic mixtures are intermediate in activity.

With acetylcholine, butyrylcholine, or tributyrin as substrate, all isomerides and the racemic mixtures inhibited the plasma pseudo-cholinesterase. With benzoylcholine as substrate (+)-, (−)- and (±)-procyclidine hydrochloride, and the atropine-like *laevo* isomerides of the methiodide and ethiodide, had an inhibitory effect, but the *dextro* forms of the methiodide and ethiodide accelerated the hydrolysis of benzoylcholine. As the concentration of these compounds was increased, the acceleration also increased, but was ultimately replaced by inhibition. Fig. 1 shows that with (+)-procyclidine methiodide the benzoylcholine curve had a peak acceleration of 34% at 5.0×10^{-4}M. Similarly, (+)-procyclidine ethiodide had a peak activity of 48% at 7.1×10^{-4}M. The racemic mix-

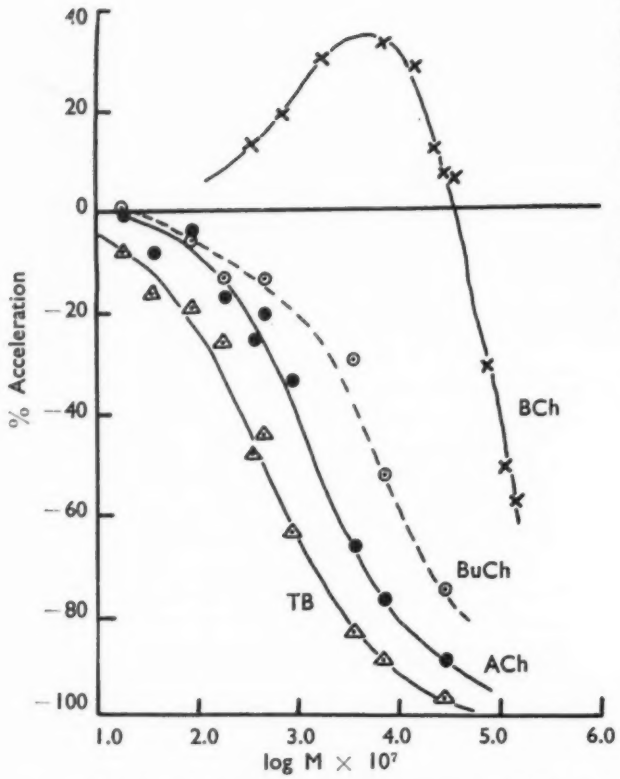

FIG. 1.—To show acceleration of pseudo-cholinesterase hydrolysis of benzoylcholine by (+)-procyclidine methiodide. Warburg respirometer. Ordinate: % acceleration of rate of hydrolysis; abscissa: log molar concentration ($\times 10^7$) of (+)-procyclidine methiodide. Peak acceleration of benzoylcholine hydrolysis of 34% at 5.0×10^{-4}M. I 50's human plasma: with benzoylcholine 1.4×10^{-2}M; with butyrylcholine 6.3×10^{-4}M; with acetylcholine 1.6×10^{-4}M.

tures of the methiodide and of the ethiodide showed slight peak accelerations which were not statistically significant. (The peak acceleration figure is taken directly from the graph.)

From these results it appeared that the accelerating effect might be associated with one or more of the following: the quaternary nitrogen in the pyrrolidine ring; the alkyl group attached to this nitrogen; or the spatial configuration of the compounds. Further compounds containing nitrogen, or showing optical activity, were therefore tested.

The Hyoscyamines.—This group was compared with the procyclidine group for four reasons: it possesses optical isomerism; its members have a pyrrolidine ring with a substituent methyl group on the nitrogen in the molecule; the nitrogen is tertiary; and the atropine activity varies in intensity among the members of the group. Here again no acceleration of the hydrolysis of acetylcholine, butyrylcholine, or tributyrin was observed in the presence of either (+)-, (−)-, or (±)-hyoscyamine, but the rate of hydrolysis of benzoylcholine was markedly increased in the presence of any of the three forms. (+)-Hyoscyamine showed an approximate peak acceleration of 80% at 2.5×10^{-3}M, (−)-hyoscyamine 100% at 2.5×10^{-3}M, and (±)-hyoscyamine (atropine) 99% at 3.2×10^{-3}M. These figures are roughly comparable. Again, increase in the concentration beyond that giving peak acceleration led to inhibition. No atropinase activity was found in human plasma. Fig. 2 shows the results obtained with atropine. Atropine was further tested on a horse serum

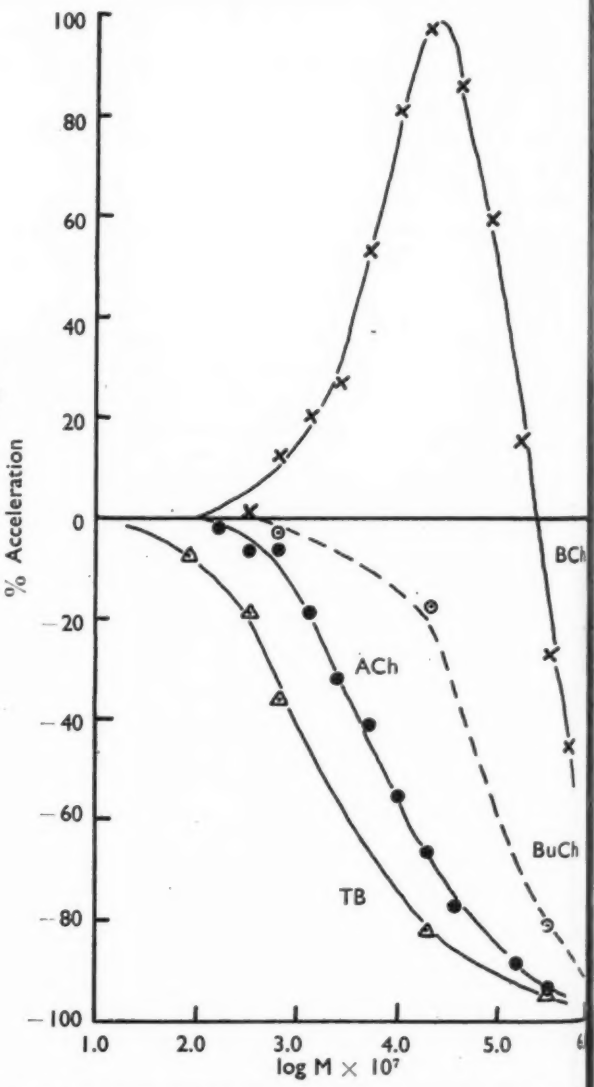

FIG. 2.—To show acceleration of pseudo-cholinesterase hydrolysis of benzoylcholine by atropine sulphate. Warburg respirometer. Ordinate: % acceleration of rate of hydrolysis; abscissa: log molar concentration ($\times 10^7$) of atropine sulphate. Peak acceleration of benzoylcholine hydrolysis of 99% at 2.5×10^{-3}M. I 50's human plasma: with benzoylcholine 5.6×10^{-2}M; with butyrylcholine 7.1×10^{-3}M; with acetylcholine 7.1×10^{-4}M.

pseudo-cholinesterase preparation, also free from atropinase activity, and the results gave a curve for benzoylcholine hydrolysis identical with the benzoylcholine curve in Fig. 2.

Local Anaesthetics.—Cocaine was investigated because the structure of part of its molecule is closely similar to that of atropine. Only (−)-cocaine hydrochloride was examined and it produced a 30% peak increase in the rate of hydrolysis of benzoylcholine chloride at 1.3×10^{-3}M. With the other three substrates the rate of hydrolysis fell with increasing drug concentration ; there was no acceleration. On the other hand, procaine hydrochloride, although a substrate for an esterase in human plasma (Kisch, Koster, and Strauss, 1943), acted only as an inhibitor with all four substrates.

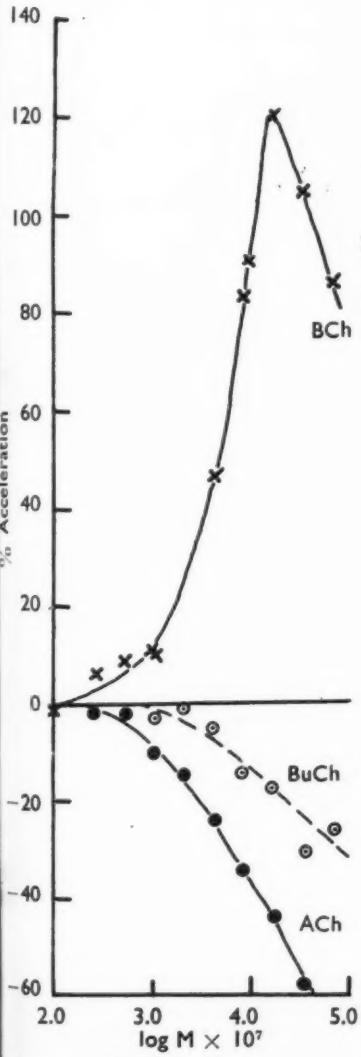

FIG. 3.—To show acceleration of pseudo-cholinesterase hydrolysis of benzoylcholine by morphine sulphate. Warburg respirometer. Ordinate: % acceleration of rate of hydrolysis; abscissa: log molar concentration ($\times 10^7$) of morphine sulphate. Peak acceleration of benzoylcholine hydrolysis of 120% at 1.8×10^{-3}M. I 50's human plasma: with benzoyl choline $\gg 10^{-2}$M; with butyrylcholine $> 10^{-2}$M; with acetylcholine 2.5×10^{-3}M.

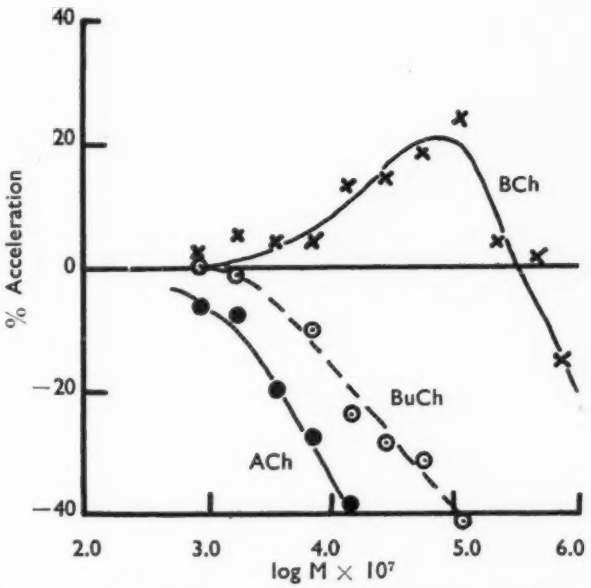

FIG. 4.—To show acceleration of pseudo-cholinesterase hydrolysis of benzoylcholine by hexamethonium iodide. Warburg respirometer. Ordinate: % acceleration of rate of hydrolysis; abscissa: log molar concentration ($\times 10^7$) of hexamethonium iodide. Peak acceleration of benzoylcholine hydrolysis of 21% at 7.9×10^{-3}M. I 50's human plasma: with benzoylcholine $> 10^{-1}$M; with butyrylcholine $\simeq 3.0 \times 10^{-2}$M; with acetylcholine $\simeq 3.0 \times 10^{-3}$M.

Analgesics.—Two analgesics were examined, morphine and thiambutene. Morphine, which contains a methyl substituted tertiary nitrogen in a piperidine ring, was a potent accelerator of the hydrolysis of benzoylcholine with a peak acceleration of 120% at 1.8×10^{-3}M. There was no acceleration of the hydrolysis of acetyl- or butyrylcholine ; tributyrin was not tested. Fig. 3 shows these effects. With nalorphine, in which the methyl group attached to the nitrogen is replaced by an allyl group, the peak acceleration of benzoylcholine hydrolysis was only 26% at 2.5×10^{-3}M.

Another analgesic with a morphine-like action, thiambutene (3-diethylamino-1 : 1-di(2′-thienyl)but-l-ene hydrochloride), which has a diethyl substituted tertiary nitrogen, inhibited cholinesterase with all four substrates.

Compounds with Two Quaternary Nitrogens.—Hexamethonium and decamethonium—both containing two trimethyl substituted quaternary nitrogens—were examined. Hexamethonium moderately accelerated the hydrolysis of benzoylcholine, with a maximum of 21% at 7.9×10^{-3}M, and inhibited the hydrolysis of acetylcholine and butyrylcholine (Fig. 4); decamethonium, on the other hand, inhibited with all three substrates.

Mephenesin.—This substance (3-(2-methylphenoxy) propane-1 : 2-diol) is unlike any of the

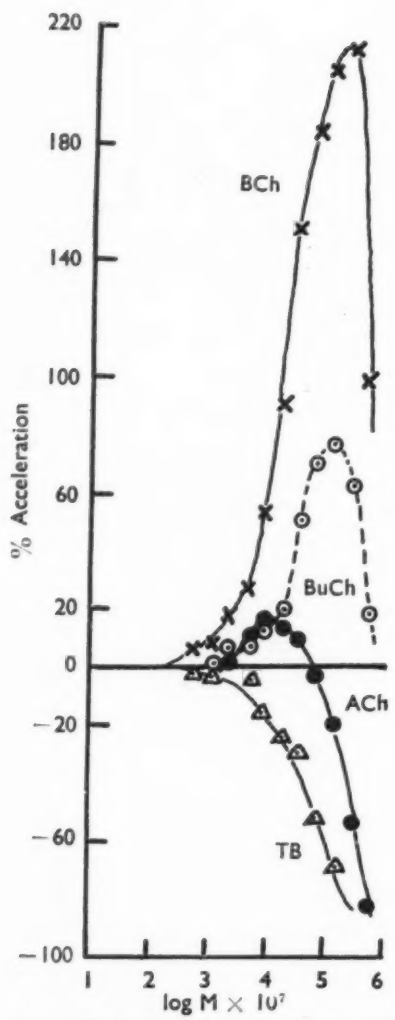

FIG. 5.—To show acceleration of pseudo-cholinesterase hydrolysis of benzoylcholine, butyrylcholine and acetylcholine by mephenesin. Warburg respirometer. Ordinate: % acceleration of rate of hydrolysis; abscissa: log molar concentration ($\times 10^7$) of mephenesin. Peak acceleration of benzoylcholine hydrolysis of 214% at 2.5×10^{-2}M; of butyrylcholine 76% at 1.6×10^{-2}M, and of acetylcholine 16% at 10^{-3}M. I 50's human plasma: with benzoylcholine $>10^{-1}$M; with butyrylcholine $>10^{-1}$M; with acetylcholine 3.2×10^{-2}M.

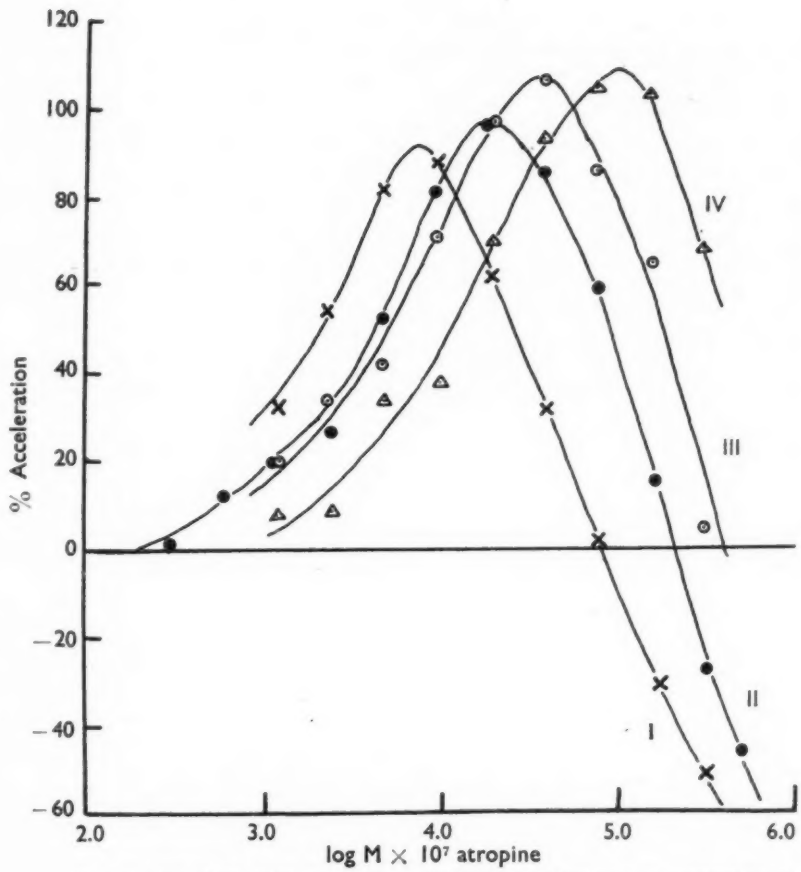

FIG. 6.—To show effect of various substrate concentrations on acceleration of pseudo-cholinesterase hydrolysis of benzoylcholine by atropine sulphate. Warburg respirometer. Ordinate: % acceleration; abscissa: log molar concentration ($\times 10^7$) of atropine sulphate. Concentrations of benzoylcholine: Curve I: 0.0075M; II, 0.015M; III, 0.03M; IV, 0.06M.

compounds so far discussed, since it contains no nitrogen, is not optically active and is not a salt. It was, however, a powerful accelerator of the hydrolysis of benzoylcholine by pseudo-cholinesterase, showing a peak acceleration of 214% at 2.5×10^{-2}M. Acceleration by mephenesin has been noted previously by Fraser (1951) and by Todrick (1954). It was, however, unique among the compounds discussed above in that it also accelerated the hydrolysis of acetylcholine (peak acceleration of 16% at 10^{-3}M) and butyrylcholine (peak acceleration of 76% at 1.6×10^{-2}M). The hydrolysis of tributyrin was inhibited (see Fig. 5).

Histidine and Folic Acid.—Aron *et al.* (1944), using a titration method for estimation, report an acceleration of the hydrolysis of acetylcholine by pseudo-cholinesterase when histidine is added to human, horse, or guinea-pig serum. This could not be confirmed using the manometric method described here. Whether this is due to a final *p*H difference, or to a difference in the duration of contact with the drug, is uncertain. No evidence of accelerated hydrolysis of the enzyme from human plasma in the presence of folic acid was obtained. The claim of Davis (1946a, b, c) limits the accelerating effect of folic acid to certain sera of dogs and human beings.

Effect on True Cholinesterase.—All the compounds examined inhibited the hydrolysis of acetyl-β-methylcholine by the true cholinesterase of human red cells.

Effect of Varying the Substrate Concentration.—To see if these acceleration effects were condi-

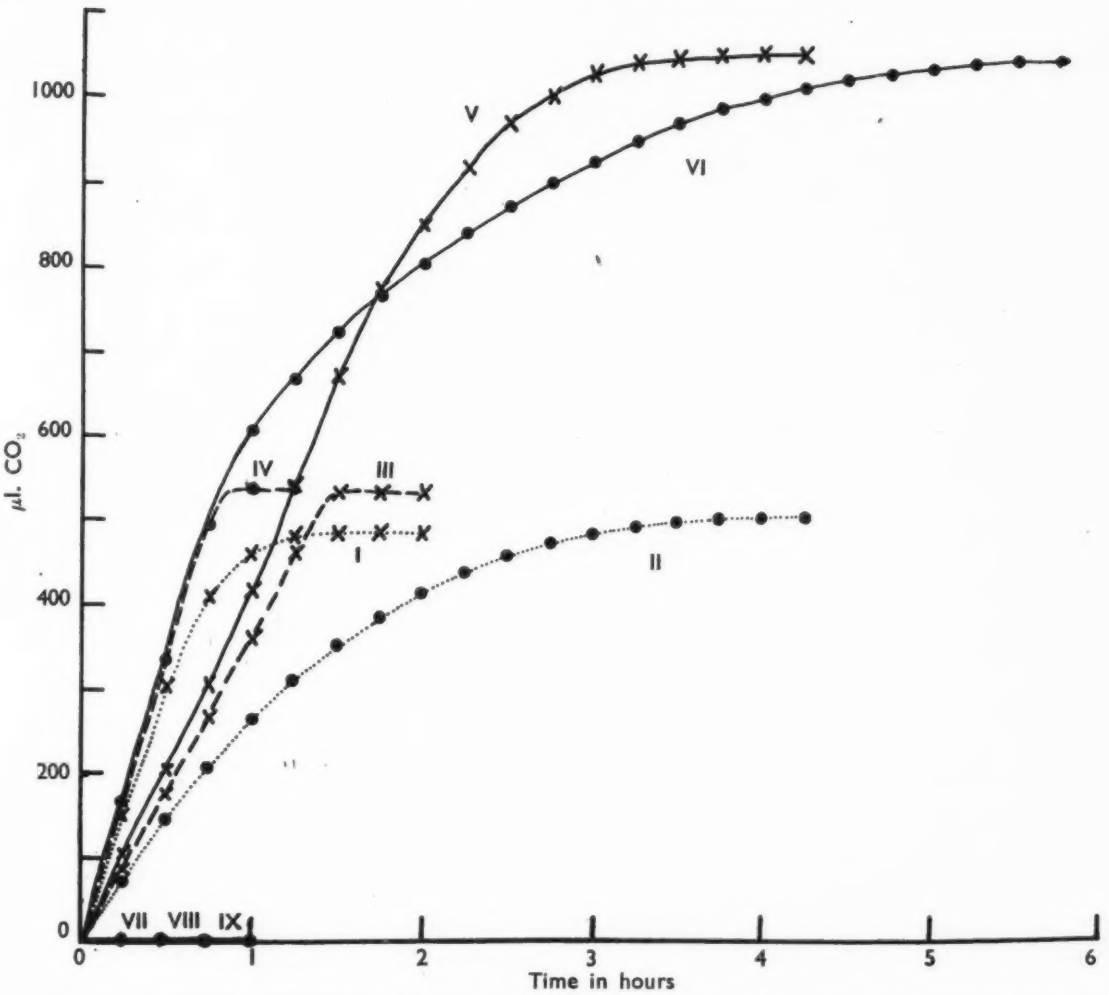

FIG. 7.—To show hydrolysis of benzoylcholine, acetylcholine and benzoylcholine+acetylcholine by human plasma in the presence and absence of atropine sulphate. Warburg respirometer. Ordinate: µl. CO_2 evolved; abscissa: time in hr. All concentrations of plasma are 1 in 15; of benzoycholine and acetylcholine 7.5×10^{-3}M, and of atropine sulphate 5×10^{-4}M. I, plasma+acetylcholine; II, plasma+acetylcholine+atropine; III, plasma+benzoylcholine; IV, plasma+benzoylcholine+atropine; V, plasma+acetylcholine+benzoylcholine; VI, plasma+acetylcholine+benzoylcholine+atropine; VII, benzoylcholine+atropine; VIII, acetylcholine+atropine; IX, plasma+atropine.

tioned by the substrate concentration, an experiment using atropine on human plasma cholinesterase was done over an eight-fold range of concentration of benzoylcholine. The results are shown in Fig. 6.

The amount of enzyme was kept constant throughout. Whatever concentration of benzoylcholine was used—from 0.0075M to 0.06M—atropine accelerated the hydrolysis. As the substrate concentration increased, the peak acceleration required higher concentrations of atropine. The nature of the effect remained the same throughout.

Effect of Substrate Combinations.—The effect of atropine on the pseudo-cholinesterase hydrolysis of benzoylcholine and acetylcholine, both singly and combined, is shown in Fig. 7; the reactions were allowed to go to completion. Control experiments with mixtures of benzoylcholine and atropine, acetylcholine and atropine, or plasma and atropine, showed no evolution of CO_2. Curves I and II show, respectively, the hydrolysis of acetylcholine in the absence and in the presence of atropine. Both reached the same maximum, but curve II took longer to reach it—that is, the hydrolysis of acetylcholine in the presence of atropine was slowed. A similar result could be obtained without atropine by reducing the amount of enzyme involved. With benzoylcholine as substrate (curves

III and IV) atropine produced the reverse effect, in that the time required to reach maximum CO_2 production was shortened. This effect could be obtained either by an increase in the rate of hydrolysis or an increase in the amount of enzyme present.

Curves V and VI show the rate of combined hydrolysis of benzoylcholine and acetylcholine in equimolar concentrations in the presence and absence of atropine. In the absence of atropine (curve V) the initial rate of hydrolysis was slightly greater than that of benzoylcholine alone, and less than that of acetylcholine. In the presence of atropine, the first part of the curve (curve VI) coincides with the benzoylcholine curve and later takes on a slope more closely approximating to the hydrolysis curve of acetylcholine in the presence of atropine. It would be reasonable to assume from these curves that, on the whole, benzoylcholine was hydrolysed preferentially to acetylcholine. Even in the mixed substrate the effect of atropine on the hydrolysis of acetylcholine is still seen, since the final attainment of complete hydrolysis was delayed by atropine.

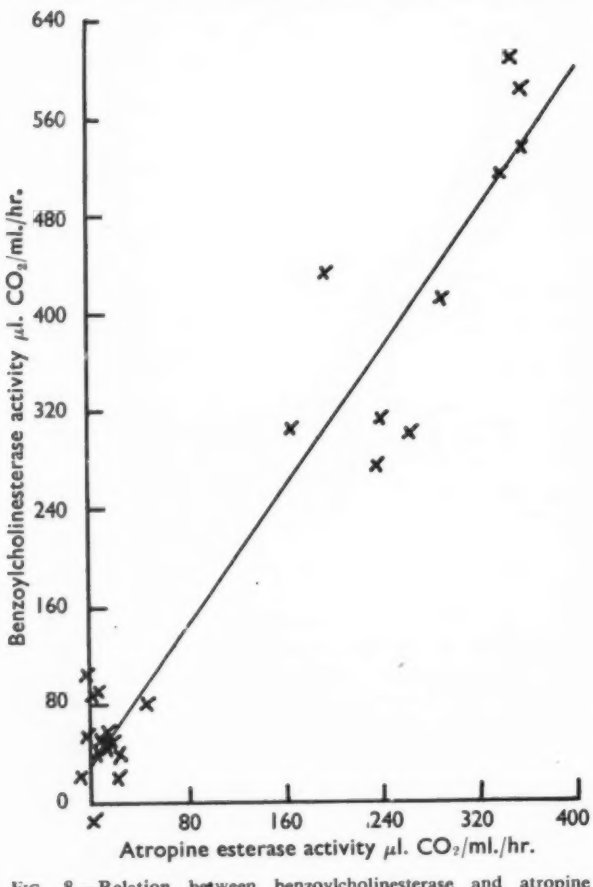

FIG. 8.—Relation between benzoylcholinesterase and atropine esterase in rabbit plasma. Warburg respirometer. Ordinate: benzoylcholinesterase activity expressed as μl. CO_2 evolved/ml. plasma/hr.; abscissa: atropine esterase activity expressed as μl. CO_2 evolved/ml. plasma/hr.

Action in the Absence of Enzyme.—In the absence of any enzyme and at high concentrations at elevated temperatures, acetylcholine and benzoylcholine may undergo spontaneous hydrolysis at a rate readily measurable in the Warburg respirometer. However, using molar concentrations of the substrate at 45° C. and a 256-fold range of atropine concentration, no acceleration of the spontaneous hydrolysis of benzoylcholine or of acetylcholine was obtained. In fact, both were slightly slowed—the hydrolysis of benzoylcholine more so than that of acetylcholine.

Rabbit Plasma.—It was of interest that, in rabbit plasma, atropine esterase activity was directly proportional to benzoylcholinesterase activity (Fig. 8). This figure gives the results obtained with the plasmas from twenty-four rabbits, and shows that in the rabbit the two enzymes were either one and the same, or occurred together in the same proportions. Ellis (1947), however, claims that they are two separable entities and has shown that, after destruction of the atropine esterase by heat, atropine inhibits the hydrolysis of benzoylcholine by benzoylcholinesterase. By incubation of rabbit liver globulin at 48° C. for 90 min. Ellis was able to destroy all the atropine esterase activity with only 20% loss of benzoylcholinesterase. Using rabbit plasma α and β globulins, as prepared by Glick, Glaubach, and Moore (1942), a temperature of 55° C. for 60 min. was necessary. Under these conditions 96% of the atropine esterase was destroyed, with the loss of only just over half of the benzoylcholinesterase activity. Using this preparation, atropine over the usual range of concentrations inhibited benzoylcholine hydrolysis by benzoylcholinesterase, similarly to Ellis's rabbit liver preparation, and at no concentration was acceleration obtained.

Purified Pseudo-cholinesterase.—Horse serum pseudo-cholinesterase was partly purified by the method of Glick *et al.* (1942). Atropine on this preparation gave an acceleration curve for benzoylcholine hydrolysis identical with that obtained from unpurified horse serum.

Atropine produced the same acceleration of benzoylcholine hydrolysis by horse serum pseudo-cholinesterase, purified by the method of Strelitz (1944), at all stages up to an estimated 5,000-fold purification.

Effects on Succinyldicholine Hydrolysis.—In the Warburg respirometer, the hydrolysis of succinyl-

dicholine by human plasma pseudo-cholinesterase was inhibited by atropine at concentrations above 10^{-4}M. Below this it had no action. At 3.2×10^{-3}M there was 50% inhibition.

Discussion

There are few reports of organic substances which activate cholinesterase. With the exception of the alcohols of Todrick *et al.* (1951), those that have appeared concern the hydrolysis of acetylcholine by pseudo-cholinesterase. In the experiments reported above, all cases of "activation" have involved benzoylcholine hydrolysis and one has further involved the hydrolysis of acetylcholine and of butyrylcholine. No activators of true cholinesterase, or of tributyrinase, have been found.

The typical action is an acceleration of the hydrolysis of benzoylcholine by pseudo-cholinesterase. As the concentration of "activator" was increased, the acceleration increased to a peak, then diminished, and was eventually replaced by inhibition.

Accelerating activity has been found in the presence of quaternary nitrogen, as in the procyclidine series; in the presence of tertiary nitrogen, in the hyoscyamine series; and in the absence of nitrogen, in mephenesin. Furthermore, activity is not necessarily associated with a particular stereochemical configuration, (+)- and (−)-hyoscyamine being active as well as (+)-procyclidine methiodide and ethiodide and (−)-cocaine. It therefore appears that this accelerating action is a function of each particular compound and is not dependent on pharmacological type or on any particular common chemical structure or configuration. The concentrations of compounds required to give these enzyme effects are, however, much higher than those required for their usual pharmacological actions.

Mechanism of Action.—In a closed system where temperature and amounts of enzyme and of benzoylcholine are kept constant there are several ways in which acceleration can be obtained. Firstly, the amount of enzyme could be increased by the activation of an inactive precursor present in the plasma. If this were so, it would be expected that the rate of hydrolysis of the other substrates would be similarly accelerated, but this has not been obtained, except with mephenesin. In addition, atropine produced acceleration curves of benzoylcholine hydrolysis which were identical with either purified or unpurified preparations of horse serum pseudo-cholinesterase. Secondly, there could be a direct action by these compounds on the substrate molecule, making benzoylcholine more susceptible to hydrolysis by pseudo-cholinesterase and the other substrates more resistant. Further, the formation of a complex between the compound and one or more of the substrates seems unlikely on chemical grounds. Additional information was provided by the experiments with rabbit plasma. This preparation contains benzoylcholinesterase instead of the pseudo-cholinesterase of human plasma, and yet no acceleration is obtained with atropine, as would be expected if a chemical complex had been formed between atropine and benzoylcholine which made it more susceptible to hydrolysis by enzyme. There does, however, appear to be some relationship between compound and substrate, since the substrate concentration experiment showed that the molar concentration of atropine at peak acceleration was, approximately, directly proportional to the molar concentration of the substrate.

Thus, by the elimination of other possibilities, it appears that these compounds probably modify the pseudo-cholinesterase molecule directly, making it more readily able to split benzoylcholine and, less readily, acetylcholine and butyrylcholine.

Another aspect of these results has been to show up the different *p*I 50 values that would be obtained with a compound of this type when tested as an *in vitro* inhibitor of pseudo-cholinesterase using benzoylcholine and butyrylcholine as specific substrates. As an example (Fig. 2), atropine sulphate has a *p*I 50 for human plasma pseudo-cholinesterase of 1.25, with benzoylcholine as substrate, but only 2.15 with butyrylcholine under identical conditions. Thus, atropine would be estimated to be eight times more powerful if butyrylcholine, rather than benzoylcholine, was the substrate. This again emphasizes the care which must be taken in the choice of substrate for anticholinesterase experiments and the necessity for the clear definition of the experimental conditions.

Summary

1. Acceleration of the hydrolysis of benzoylcholine by pseudo-cholinesterase *in vitro*, without acceleration of the hydrolysis of butyrylcholine or acetylcholine, is described.

2. Compounds shown to have this action were: (+)-procyclidine methiodide, (+)-procyclidine ethiodide, (+)-, (−)-, and (±)-hyoscyamine, (−)-cocaine, morphine, nalorphine, and hexamethonium. Allied compounds which did not possess this action were: (+)- and (−)-procyclidine hydrochloride, (−)-procyclidine methiodide and ethiodide, procaine, thiambutene, and decamethonium.

3. Mephenesin accelerated the hydrolysis of acetylcholine and butyrylcholine, as well as that of benzoylcholine.

4. None of these compounds accelerated the hydrolysis of tributyrin by tributyrinase, or that of acetyl-β-methylcholine by true cholinesterase.

5. It is postulated that this action is produced by a modification of the pseudo-cholinesterase molecule, making it more able to hydrolyse benzoylcholine and less able to hydrolyse acetylcholine and butyrylcholine.

6. The concentrations required are well above pharmacological levels.

7. It is emphasized that care must be taken in interpreting the results of *in vitro* tests for pseudocholinesterase inhibitors when employing either benzoylcholine or butyrylcholine as substrates.

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ANALYSIS OF THE CENTRAL RESPIRATORY ACTION OF NALORPHINE IN DECEREBRATE DOGS

BY

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Nalorphine has enjoyed widespread use as a counter measure in morphine-induced respiratory depression (Unna, 1943; Hart and McCawley, 1944). There is also convincing evidence that nalorphine can exert an ameliorating effect on respiratory depression induced in human beings by various barbiturates (Dulfano, Mack, and Segal, 1953), and can counteract depression of respiratory activity produced in experimental animals by pentobarbitone sodium or chloralose-urethane (Vivante and Kao, 1953; Belford and Kao, 1954; Vivante, Kao, and Belford, 1954), and other compounds (Costa and Bonnyçastle, 1955). Thus, from the available evidence, it seems justifiable to postulate that nalorphine acts in such a way as to stimulate depressed respiratory centres* regardless of the depressant drug. Nalorphine also stimulates respiration in decerebrate dogs, and its site of action is not on the peripheral chemoreceptors (Vivante *et al.*, 1954).

A respiratory stimulant or depressant may act directly on the respiratory centres, or indirectly by changing the sensitivity of the centres to physiological stimuli, such as carbon dioxide tension. These effects may depend on, or be independent of, the molecular structure of the drug. It is the purpose of this paper to determine whether nalorphine operates through a direct stimulating action, or by sensitizing the respiratory centres, or by both mechanisms.

METHODS

Mongrel dogs of both sexes were used. Midbrain decerebration (Kao, Schlig, and Brooks, 1955) was performed under thiopentone sodium (25 mg./kg. i.v.) anaesthesia, supplemented, when necessary, with ether. A tracheal cannula was inserted and connected to a Douglas valve, the inlet of which was connected to a Douglas bag containing a CO_2-O_2 gas mixture and the outlet to a gasometer for measurement of the total ventilation. Respiratory rate was counted by observing the movement of the hand on the dial of the gasometer. Arterial blood samples were collected anaerobically from one femoral artery for both *p*H and plasma CO_2 determinations. The plasma total CO_2 was determined by means of a Van Slyke manometric apparatus, and the *p*H was measured with a Cambridge research-model *p*H meter using a water jacket (38° C.) microcondenser-type electrode. The P_{CO_2} was calculated from the Henderson-Hasselbalch equation.

A control period of 30 min. was allowed before the CO_2-O_2 mixture was given. After the ventilation had reached a steady state while the animal was breathing the CO_2-O_2 mixture, nalorphine (30 mg./kg. i.v.) was administered. Ten min. later the dog was given room air to breathe. Additional CO_2 inhalation experiments were performed on intact dogs anaesthetized with pentobarbitone sodium in an initial dose of 30 mg./kg. i.v., followed by smaller doses to produce deep respiratory depression.

RESULTS

The Respiratory Effect of CO_2 Inhalation in Dogs Anaesthetized with Pentobarbitone

The respiratory response of an anaesthetized dog to CO_2 inhalation is shown in Fig. 1, in which ventilation in l./min. BTPS (Committee on Nomenclature of Respiratory Physiology, 1950) is plotted as a function of time. At zero time, CO_2-O_2 mixtures were given. This figure shows that the same gas mixture did not necessarily initiate similar respiratory responses. This is better shown in Fig. 2, in which the response to CO_2 inhalation (5% CO_2 in O_2) is plotted against P_{CO_2} in mm. Hg. It is clear that in an anaesthetized dog ventilation was dependent on the level of anaesthesia. In deep anaesthesia the ventilation was less than during light anaesthesia. Correspondingly, the arterial P_{CO_2} was high during deep anaesthesia, as is to be expected during respiratory depression.

*The term respiratory centres as used here includes the central chemoreceptors which are sensitive to *p*H and P_{CO_2} and are located in the medullary tissue near the inspiratory and expiratory centres (Grodins, Gray, Schroeder, Norins, and Jones, 1954).

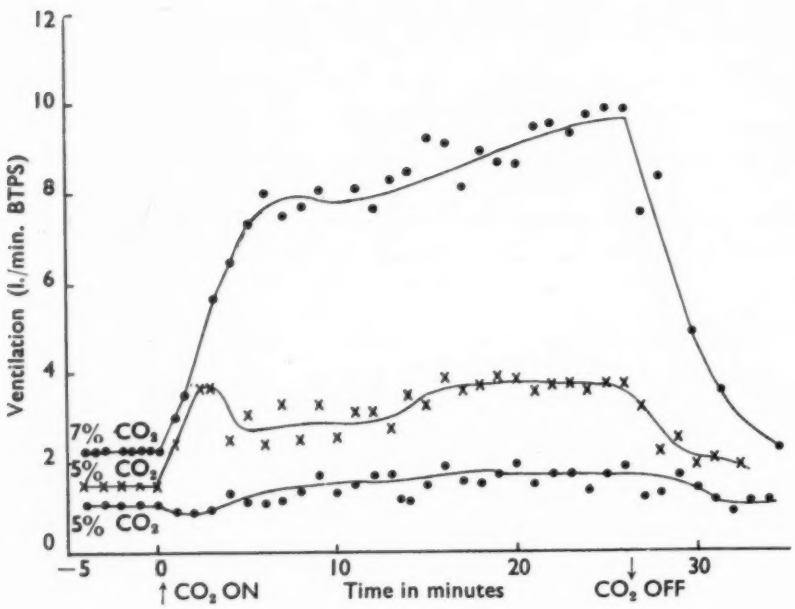

FIG. 1.—Dog, 9.3 kg., pentobarbitone anaesthesia; administration of 5% (1.15 p.m., bottom curve), 5% (2.15 p.m., middle curve) and 7% (3.15 p.m., top curve) CO_2 in the inspired air caused an increase of ventilation over the resting (30 min.) rate (only the last 5 min. of which is shown). Note: (a) no return to original resting value in each instance and (b) a similar but quantitatively different response to the administration of 5% CO_2.

The difference in response to the same percentage of CO_2 in the inspired air in an animal at different depths of anaesthesia is revealed by the different slopes of the family of lines in Fig. 2: the slopes were higher when anaesthesia was light, and vice versa. Another interesting point shown in Fig. 2 is that administration of a CO_2-O_2 mixture to a dog whose respiration is already deeply depressed by anaesthesia depresses respiration still further. This is to be expected, since a high concentration of O_2 was given with the CO_2, and such deeply anaesthetized dogs depend for the maintenance of their ventilation on the sensitivity of their peripheral receptors to O_2 lack.

It seems obvious from these observations that the sensitivity of the respiratory centres is affected by the level of anaesthesia. Hence the use of anaesthetized animals to test respiratory sensitivity to various agents is inadvisable. It is very difficult to maintain such animals at the same level of sensitivity. During anaesthesia the acid-base balance of dogs is dependent on respiratory activity, and the P_{CO_2} and the H^+ concentration of the arterial blood vary in the same direction (Fig. 3). Arterial puncture was carried out in one dog before anaesthesia, and it was found that the P_{CO_2} was 53.6 mm. Hg and the H^+ concentration was 38.5 mμM/l. After anaesthesia they were 70.5 and 51.9 respectively.

Similar observations were made on five anaesthetized dogs.

The Respiratory Effect of CO_2 Inhalation in Decerebrate Dogs

Eighteen tests were carried out in six decerebrate dogs to determine the sensitivity of their respiratory centres to CO_2 inhalation. The response of ventilation to CO_2 inhalation is shown in Fig. 4. Within the time of observation a reasonably steady state was attained. Fig. 5 shows the relation between ventilation and P_{CO_2} in mm. Hg during the steady state. A regression line (a) was fitted to the data, employing

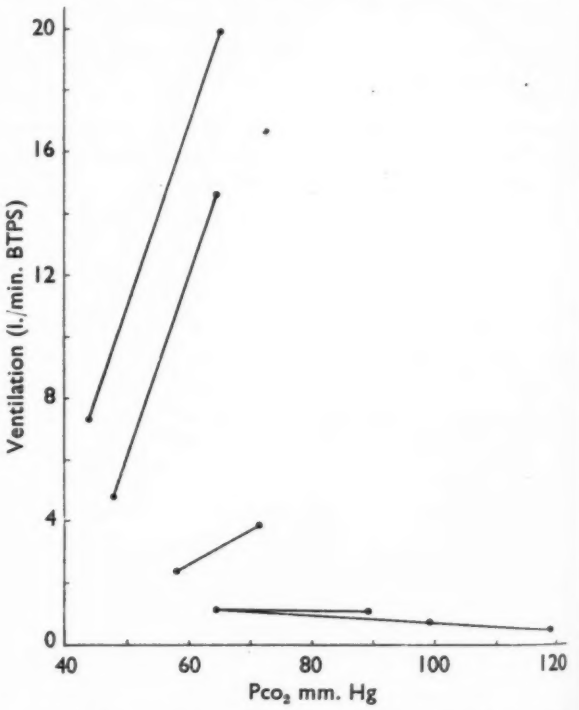

FIG. 2.—Dog, 9.6 kg.; pentobarbitone (40 mg. kg.) anaesthesia. The relation between ventilation and arterial blood P_{CO_2} at various depths of depression. The percentage (5%) of inhaled CO_2 did not vary. The slopes of the lines indicate the different sensitivity of the respiratory centres to CO_2 stimulation.

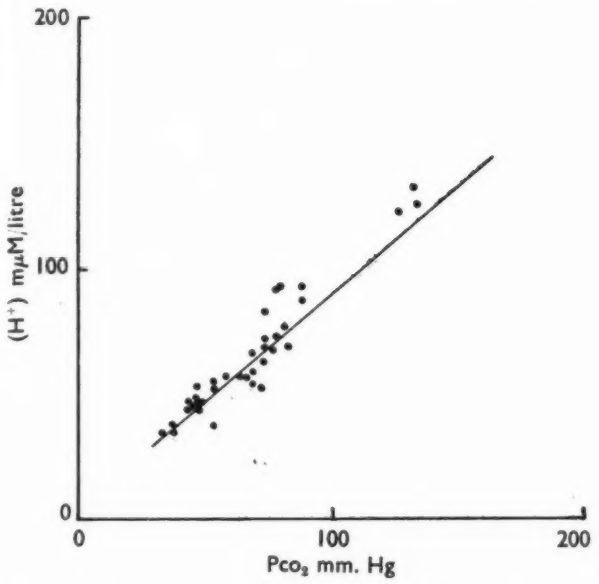

FIG. 3.—The steady-state relation between arterial blood H^+ concentration and P_{CO_2} in five dogs (36 determinations) anaesthetized with pentobarbitone.

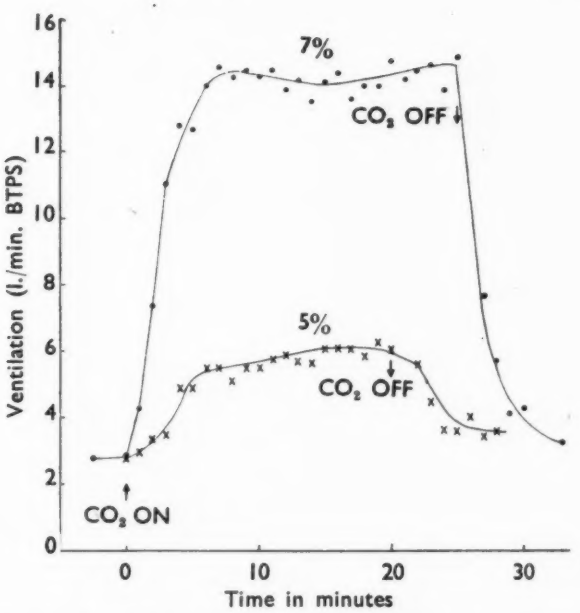

FIG. 4.—Dog, 8.2 kg. Decerebrate. Ventilatory response to 5% and 7% CO_2 inhalation. Note that the resting levels of ventilation returned to the same value between gas administrations.

an analysis of covariance (Snedecor, 1948), and it has the following equation:

$$\text{Ventilation (l./min. BTPS)} = 0.39\ (\pm\ 0.08)\ P_{CO_2}\text{ mm. Hg} - 10.32 \quad (1)$$

with $r = 0.80$. Both the slope, 0.39, and the correlation coefficient, 0.80, are significant at the 1% level of probability. In contrast to the changes in sensitivity of the respiratory centres to CO_2 in the anaesthetized dogs, the respiratory centres of decerebrate dogs had a reasonably constant sensitivity.

The Effect of Nalorphine on the Respiratory Response to CO_2 Inhalation in Decerebrate Dogs

Thirteen tests were performed on five decerebrate dogs to investigate the effect of nalorphine on the sensitivity of the respiratory centres to CO_2 inhalation.

The ventilatory response to CO_2 inhalation and to nalorphine administration is shown in Fig. 6. CO_2 was given to this dog in the following sequence, 5%, 3%, and 7%. Nalorphine (30 mg./kg. i.v.) was administered after a steady state was reached in each instance. Fig. 6 shows that nalorphine apparently causes a similar response irrespective of the percentage of CO_2 inhaled. This statement is supported by the analysis of the data in Fig. 7, in which the ratio of the increments in ventilation after the first nalorphine administration to the ventilation after the subsequent nalorphine injection is plotted against the percentage of the inhaled CO_2. The regression line fitted to this group of data has a slope of 0.09 with an error of 0.13. That the slope does not significantly differ from zero indicates that the increase in ventilation after the injection of nalorphine is independent of the CO_2 administered.

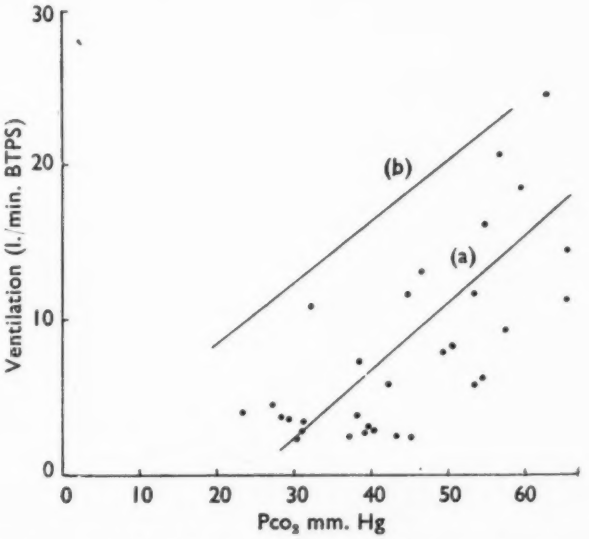

FIG. 5.—The relation between ventilation and P_{CO_2} in 6 decerebrate dogs during the steady-state condition of CO_2 inhalation (line (a) with dots). Line (b) represents the same relationship when in addition to CO_2 inhalation nalorphine was administered.

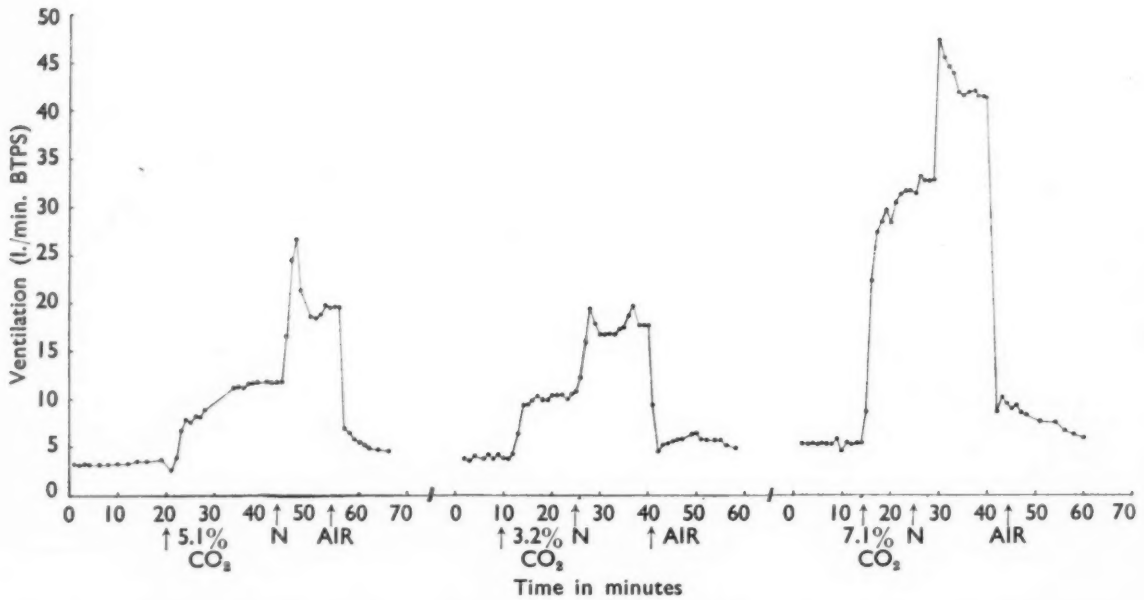

FIG. 6.—Dog, 13.6 kg. Decerebrate. Ventilatory responses to various CO_2 mixtures. At N nalorphine was administered. The animal was given room air to breathe at AIR. One hour elapsed between experiments. There is no statistical (*vide infra*, Fig. 7) difference in response to nalorphine when its administration is superimposed on each of the gas mixture inhalations.

Ventilation after nalorphine injection is plotted as a function of P_{CO_2} in mm. Hg (Fig. 5, line b). The slope of the line is 0.37 with $r=0.85$, and the complete equation expressing ventilation and P_{CO_2} is:

Ventilation (l./min. BTPS) = 0.37 ($\pm$0.18) P_{CO_2} mm. Hg + 0.45 (2)

The slope in this equation is significantly different from zero, but does not differ from the slope in equation (1) ($b=0.39$; $P>0.50$). In other words, the two lines expressed by equations 1 and 2 are parallel. This indicates that the sensitivity of the respiratory centres to CO_2 inhalation is not altered on the administration of nalorphine, and suggests that the nalorphine acts independently of changes in CO_2 tension.

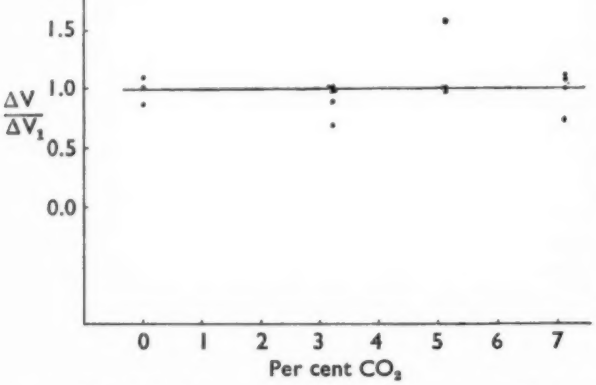

FIG. 7.—The relation between the ratio of the increments in ventilation after the first nalorphine administration to that after the subsequent nalorphine injections (with or without CO_2 inhalation) and the percentage of CO_2 in the inspired gas mixture. ΔV_1 = the increment of ventilation after the first injection of nalorphine. ΔV = the increment of ventilation after the succeeding injection of nalorphine. The line fitted to this group of data has a slope which is not significantly different from zero.

DISCUSSION

It seems appropriate to define the term sensitivity, since different authors may attach different meanings to it. If a stimulus is applied to a system and a response produced, the magnitude of the response is a function of two factors: (1) the magnitude of the stimulus and (2) the sensitivity of the system in question. If the sensitivity of a system is constant, the response plotted as a function of stimulus must be rectilinear. That is, the slope of the line expressing the relation between response and stimulus is constant. If, on the other hand, the slope changes, this implies that the sensitivity of the system is altered. The altered slope may be such that the whole line is shifted but is still rectilinear, or it may change according to the magnitude of the stimulus; in the latter event a curvilinear relation between response and stimulus would be expected. The curvature of the lines may increase or decrease according to the increase or decrease in the sensitivity of the system. Hence, the term sensitivity is here defined

as the magnitude of the change in response as a result of the change of the stimulus, or to express it symbolically:

$$\text{sensitivity} = \frac{\Delta R}{\Delta S}$$

where ΔR is the change in response and ΔS is the change in stimulus.

If in any particular situation a certain response is elicited after a stimulus is applied, and a definite sensitivity of the system is indicated, then upon adding another stimulus—as, for example, nalorphine during CO_2 inhalation—the change of sensitivity due to the second stimulus can be determined.

As the two lines in Fig. 5 are parallel, it is obvious that the respiratory centre was equally sensitive to CO_2 before and after nalorphine. The line expressing the relation between ventilation and P_{CO_2} after nalorphine injection is merely shifted upwards. It seems justifiable, therefore, to conclude that nalorphine does not change the sensitivity of the respiratory centres to CO_2 inhalation, but merely produces an additive effect. It may further be concluded that nalorphine has its own action, which is entirely independent of the carbon dioxide tension.

Summary

1. Dogs anaesthetized with pentobarbitone show respiratory acidosis, and the respiratory response to inhalation of CO_2 alters with the depth of anaesthesia.

2. The respiratory response to CO_2 inhalation is more constant in decerebrate dogs than in dogs anaesthetized with pentobarbitone. The relation between ventilation and arterial P_{CO_2} is expressed by a regression line which has a slope of 0.39.

3. In decerebrate dogs following administration of nalorphine in addition to CO_2, the relation between ventilation and arterial P_{CO_2} is expressed by a regression line which has a slope of 0.37; the latter is not significantly different from the value 0.39 ($P>0.50$).

4. It is concluded that nalorphine does not alter the sensitivity of the respiratory centres to CO_2 inhalation in decerebrate dogs. The mechanism of its respiratory stimulant activity is briefly discussed.

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ANTIPYRETIC AND ANALGESIC PROPERTIES OF TWO HYDROXY*ISO*PHTHALIC ACIDS

BY

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4-Hydroxy*iso*phthalic acid (4-HIPA) was first described by Barth (1872). Idris Jones and his co-workers (Reports of Chemistry Research Board, 1952, 1953) identified 4-HIPA as the main constituent of the "brown dust" residues from the sublimation of salicylic acid during manufacture. They suggested that 4-HIPA should be examined for similar pharmacological properties to salicylic acid and they provided material for this purpose. Pharmacological examination showed that this compound indeed possessed potent antipyretic and mild analgesic properties, which led us to examine also 2-hydroxy*iso*phthalic acid (2-HIPA), another by-product of the Kolbe-Schmitt process for the manufacture of salicylic acid. The results have been briefly reported (Chesher, Collier, Robinson, Taylor, Hunt, Idris Jones, and Lindsey, 1955). The present paper describes in detail the pharmacological work on both compounds.

METHODS

Antipyretic Tests.—Himalayan rabbits of either sex, weighing between 2.0 and 3.5 kg., which had been deprived of food and kept in an even laboratory temperature overnight, were placed in neck stocks in individual boxes. Rectal thermocouples were inserted to a depth of about 7 cm. Galvanometer readings were taken half-hourly to a total of 12 readings over a period of 5½ hr. After the third reading, each rabbit received intravenously per kg. 1 ml. saline containing 0.5 μg. of pyrogen prepared from *Proteus vulgaris* in the same way as fraction F68 of Miles (1951). After the fifth reading, suspensions of 4-HIPA, 2-HIPA, or aspirin were injected intraperitoneally, except in the control group. In order to reduce the readings in all rabbits to a comparable basis, each was expressed as the difference in °C. from the mean of the first three readings in the same animal.

Analgesic Tests.—Young Wistar rats of either sex within the weight-range 50–200 g. were used. Experiments reported below indicated that the same animal might safely be used on up to five occasions not more than twice weekly. In each experiment rats of about equal weight were distributed in dosage groups of ten or more and one group received a control solution, either of gum acacia-saline or of 5% sodium dihydrogen phosphate adjusted with N-HCl to the same *p*H as 4-HIPA suspensions (approximately 2.9). By means of a modified analgesiometer of Green, Young, and Godfrey (1951), in which the horizontal syringe was propelled by hand instead of by motor, we determined the pressure, applied to the tip of the rat's tail, required to elicit a squeak. This is called the squeak-threshold. An analgesic response was taken as a raising of the squeak-threshold to at least twice the geometric mean of the control group.

Local Anaesthetic Test.—Aqueous solutions of drugs were administered intradermally to guinea-pigs and the proportions of responses to needle pricks at the site of injection observed, according to the method of Somers and Edge (1947).

Toxicity Tests.—Albino male mice weighing 18–24 g. and male Wistar rats of 50–120 g. were used. In acute tests deaths were counted after seven days. In chronic tests drugs were mixed in a dry powdered diet (M.R.C. No. 41) and animals were weighed weekly. Blood haemoglobin, red cells, and white cells were estimated by conventional methods and prothrombin in plasma, diluted to 12.5% in saline, by the method of Quick, Stanley-Brown, and Bancroft (1935).

Administration of Drugs.—4-HIPA, 2-HIPA, aspirin, salicylic acid, salicylamide, and phenacetin were administered in suspension in 5% gum-acacia in 0.9% saline. Calcium 4-hydroxy*iso*phthalate, calcium aspirin, procaine hydrochloride, codeine, codeine phosphate, morphine hydrochloride, pethidine hydrochloride, nalorphine hydrobromide, pentobarbitone sodium, and thiopentone sodium were given in aqueous solutions and methylpentynol in saline. When salts were used weights of drugs are expressed as the active acid or base.

RESULTS

Antipyretic Experiments in Rabbits

The antipyretic activity of 4-HIPA is shown in the experiment illustrated in Fig. 1. Twenty

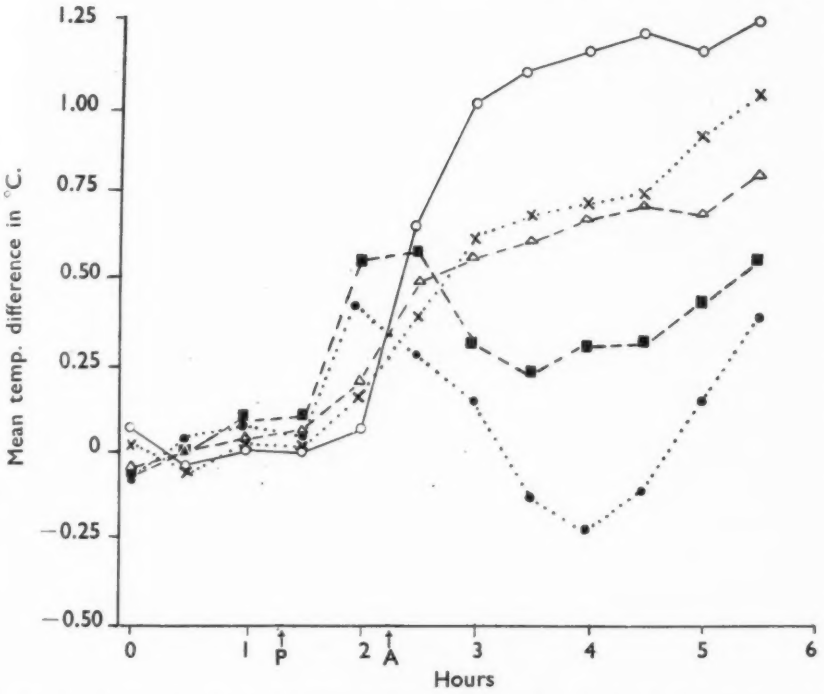

FIG. 1.—Antipyretic effects of 4-HIPA and aspirin on rabbits treated with *Proteus* pyrogen. Pooled results of a twin cross-over test in 20 rabbits. At P, 0.5 μg. pyrogen i.v.; at A, antipyretic injected i.p. O–O = pyrogen alone; × ··· × = pyrogen + 4-HIPA 22.2 mg./kg.; Δ–Δ = pyrogen + aspirin 22.2 mg./kg.; ■–■ = pyrogen + aspirin 66.7 mg./kg.; ● ·· ● = pyrogen + 4-HIPA 66.7 mg./kg.

rabbits received pyrogen; 2 groups of 4 then received 4-HIPA or aspirin, either 66.7 or 22.2 mg./kg.; the remaining 4 animals being controls. Three days later the rabbits were again treated, except for one moribund animal which had received the higher dose of aspirin. The same rabbits were used as controls on both occasions, their mean maximum temperature rises being 1.26° C. on the first day and 1.34° C. on the second. On the second day of test the group that had received the higher dose of aspirin now received the higher dose of 4-HIPA, and so on.

The corresponding temperature differences obtained with the same dose of drug were pooled and their means plotted against time (Fig. 1). The temperature in all groups began to rise soon after the administration of pyrogen. In the control group, the temperature reached an approximate plateau within 2 hr.; in rabbits receiving 4-HIPA or aspirin as well as pyrogen, the rise was wholly or partly suppressed and sometimes replaced by a fall.

The antipyretic response of each rabbit was derived from its lowest temperature between 2 and 4 hr. after giving the pyrogen. These responses in the various dosage groups were compared by means of *t* tests. At the 66.7 mg./kg. dose level the response to 4-HIPA was not quite significantly greater than the response to aspirin ($P = 0.1–0.05$); at the 22.2 mg./kg. dose level the difference in mean responses to the two drugs was negligible. In further comparative tests, 24 rabbits received pyrogen; 8 of these then received 66.7 mg./kg. of 4-HIPA, and 8 a similar dose of aspirin. The mean lowest temperature difference after 4-HIPA was 0.14° C. and after aspirin 0.11° C., while the mean maximum rise in the controls was 1.46° C. We conclude that 4-HIPA and aspirin possess a similar degree of antipyretic activity.

In a further experiment, 12 rabbits were treated with pyrogen. Four of these then received 200 mg./kg. 2-HIPA and another four an equal dose of 4-HIPA. Both groups showed a comparable antipyretic response.

Analgesic Experiments in Rats

Single Doses.—Single large but not lethal doses of 4-HIPA, given intraperitoneally to rats, raised their squeak-thresholds significantly above those of control animals receiving buffer solution at the same *p*H. The analgesia was unaccompanied by loss of righting reflex, drowsiness or other visible side-effects. Although we failed to detect anal-

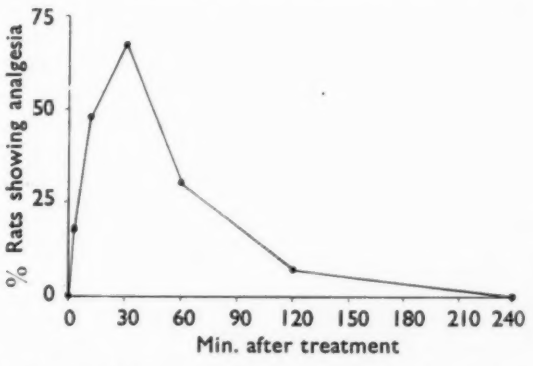

FIG. 2.—Time-course of analgesia in rats after intraperitoneal injection of 450 mg./kg. 4-HIPA.

gesia in most rats when 4-HIPA was given entirely by the oral or subcutaneous routes, the proportion of analgesic responses could be increased by giving a large oral dose, together with a just effective intraperitoneal dose.

In experiments on the time-course of analgesia, 450 mg./kg. 4-HIPA was administered intraperitoneally to rats, and at various times afterwards groups of 20 were tested by analgesiometer. At 3 min. after treatment the squeak-threshold had risen in some animals; at 30 min. the proportion of rats showing analgesia reached its peak, and by 4 hr. the response had disappeared (Fig. 2). In animals destroyed 6 hr. after treatment no traces of 4-HIPA could be detected in the peritoneal cavity. In further analgesic experiments the squeak-threshold was measured at 30 min. after treatment.

TABLE I

POTENCIES AND TOXICITIES OF SOME ANALGESICS IN RATS

Codeine administered subcutaneously in aqueous solution, remaining drugs intraperitoneally in gum acacia-saline

Drug	Potency		Toxicity	
	No. of Rats	ED50 (Fiducial Limits)	No. of Rats	LD50 (Fiducial Limits)
4-HIPA ..	968	315 (279 to 354)*	79	1,071 (968 to 1,185)*
2-HIPA ..	110	356 (286 ,, 443)*	20	925 (892 ,, 957)†
Codeine ..	98	11·5 (9·3 ,, 13·7)†	35	372 (297 ,, 447)†
Aspirin ..	—	—	76	541 (485 ,, 603)*
Salicylamide	60	175 (165 ,, 187)†	40	592 (573 ,, 610)†

* Computed. † Graphical estimate.

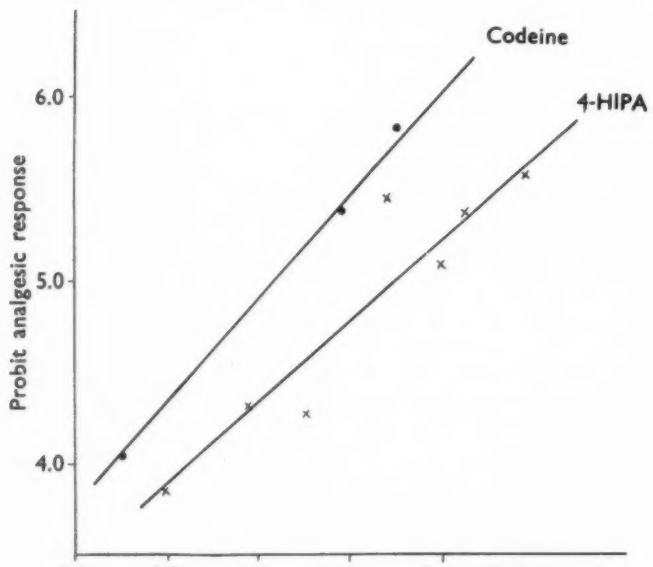

FIG. 3.—Log dose-probit response lines obtained by pressure analgesiometer in young rats.

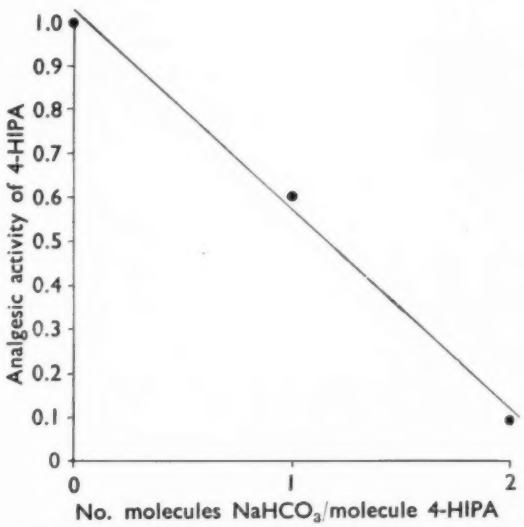

FIG. 4.—Effect of reaction with sodium bicarbonate on analgesic activity of 4-HIPA. Activity of 4-HIPA alone = 1.0.

In Table I are expressed the analgesic activities of 4-HIPA, 2-HIPA, codeine and salicylamide. The ED50 of salicylamide was identical with that found by Way, Takemori, Smith, Anderson, and Brodie (1953), who used a similar technique. 2-HIPA and codeine resembled 4-HIPA in lack of visible side-effects in the rat. When codeine and 4-HIPA were administered intraperitoneally, their probit-log dose lines were approximately parallel (Fig. 3) and we therefore compared directly their analgesic activities by this route. From experiments in 381 rats, 4-HIPA possessed 4.16% the potency of codeine (fiducial limits, 3.21 to 5.54). We did not compare 4-HIPA directly with salicylates, because aspirin and salicylic acid in just tolerated doses (300 and 400 mg./kg. respectively) failed to produce a significant analgesic response, and salicylamide caused sleepiness as well as analgesia, as described by Berger (1954).

4-HIPA is relatively insoluble in water (1 in 5,000 at 20° C.); but the product of mixing sodium bicarbonate and 4-HIPA in cold water in proportion of 2 molecules $NaHCO_3$ to 1 molecule 4-HIPA was more soluble. When such a solution, of *p*H 6.8, was injected intraperitoneally, its analgesic activity and toxicity were very low, the activity being about one-tenth that expected from the 4-HIPA added to the reaction mixture. By the intravenous route the solution was also of low activity.

In order to explore this effect still further, we examined the analgesic potency of an equimolecular mixture of $NaHCO_3$ and 4-HIPA in water, which formed a suspension of *p*H 4.4. Its analgesic activity was about 60% that expected from the 4-HIPA added to the reaction mixture. The results of these experiments are expressed in Fig. 4.

Repeated Administration.—Groups of 10 rats were dosed intraperitoneally twice weekly for 3 weeks with 450 or 150 mg./kg. 4-HIPA, with 15 or 5 mg./kg. codeine or with 10 ml./kg. saline. After each administration, rats were tested by analgesiometer. Over this period there was no significant change in the analgesic response to either drug. After the 4th dose, 1 rat receiving 450 mg./kg. 4-HIPA died.

During a period of 5 weeks, 8 intraperitoneal doses of 200 mg./kg. 4-HIPA or aspirin or of 10 ml./kg. phosphate buffer at *p*H 2.9 or saline were administered to groups of 10 rats. During these weeks, 1 rat receiving aspirin and 1 receiving saline died. At the end of the period, all survivors were treated intraperitoneally with 15 mg./kg. codeine and tested by analgesiometer. Four days later the experiment was repeated, using 450 mg./kg. 4-HIPA instead of codeine. Both control groups gave normal responses to both analgesics, but the drug-treated animals showed reduced responses. At the end of this experiment the animals were destroyed. Post-mortem observations are discussed below under the heading of subacute toxicity.

Local Anaesthetic Experiments

To examine the possibility that the calcium salt of 4-HIPA possessed local anaesthetic activity, we compared it with procaine hydrochloride and calcium aspirin, using a calcium chloride solution of equivalent calcium content as control. In these experiments, calcium 4-HIPA and calcium aspirin showed significant but very slight local anaesthetic activity, which was about 3% that of procaine.

5% solutions of the calcium salt of 4-HIPA and of calcium aspirin caused local necrosis after intradermal injection.

Toxicity

Acute.—The acute toxicities to rats of 4-HIPA, 2-HIPA and various other analgesics with which they were compared are summarized in Table I.

Subacute.—The rats, which had received 8 doses of 4-HIPA, aspirin or control solutions during 5 weeks in the experiment described above, were destroyed and examined *post mortem*. In all drug-treated animals, the livers were pale and swollen and the liver capsules and other parts of the peritoneum were thickened and adherent. There was also some blood-stained fluid in the peritoneal cavity. On histological examination of the livers, the only change detected was capsular hypertrophy.

Chronic.—In a preliminary chronic toxicity test, over a period of 14 weeks, 2 of 10 mice receiving 0.5% and 3 of 10 receiving 1% 4-HIPA in their diet died. In corresponding dosage groups receiving aspirin, there were 3 and 2 deaths respectively.

Rats were maintained for 26 weeks on diets containing 0.2 or 0.1% 4-HIPA or aspirin. All animals survived; but towards the end of this period one rat on 0.2% 4-HIPA lost weight and showed abdominal enlargement. Blood examinations (Table II) did not show any substantial difference between treated and control rats, although the white cell counts were low throughout. The differences in prothrombin time, however, between untreated rats and those receiving 0.2% 4-HIPA ($P=0.1$) or aspirin ($P=0.2$) approached significance. The growth curves of the untreated rats and those receiving the higher percentages of 4-HIPA or aspirin are shown in Fig. 5, from which it will be seen that aspirin, and to a lesser extent 4-HIPA, slightly depressed the rate of growth.

Histological examination of sections of brain, stomach, small and large intestine, liver, thyroid,

TABLE II

MEAN VALUES OBTAINED FROM BLOOD EXAMINATION OF RATS WHICH HAD RECEIVED 4-HIPA OR ASPIRIN IN THEIR DIET FOR 26 WEEKS

L=lymphocytes; M=monocytes; N=neutrophils; E=eosinophils; B=basophils

% Drug in Diet	% Haemoglobin	Erythrocytes per $mm^3 \times 10^6$	Leucocytes per $mm^3 \times 10^3$	Differential Counts %				Prothrombin Time in Sec.
				L and M	N	E	B	
0·2 4-HIPA	108	8·6	4·5	73·55	23·40	3·00	0·05	28·3
0·1 ,, ...	108	9·1	5·0	73·80	24·00	2·20	0·00	26·1
0·2 aspirin	106	8·6	4·4	74·60	22·25	3·15	0·00	28·2
0·1 ,, ...	113	8·7	5·0	81·90	15·15	2·90	0·05	27·1
Untreated controls ..	110	8·4	4·2	79·80	18·10	2·05	0·05	25·2

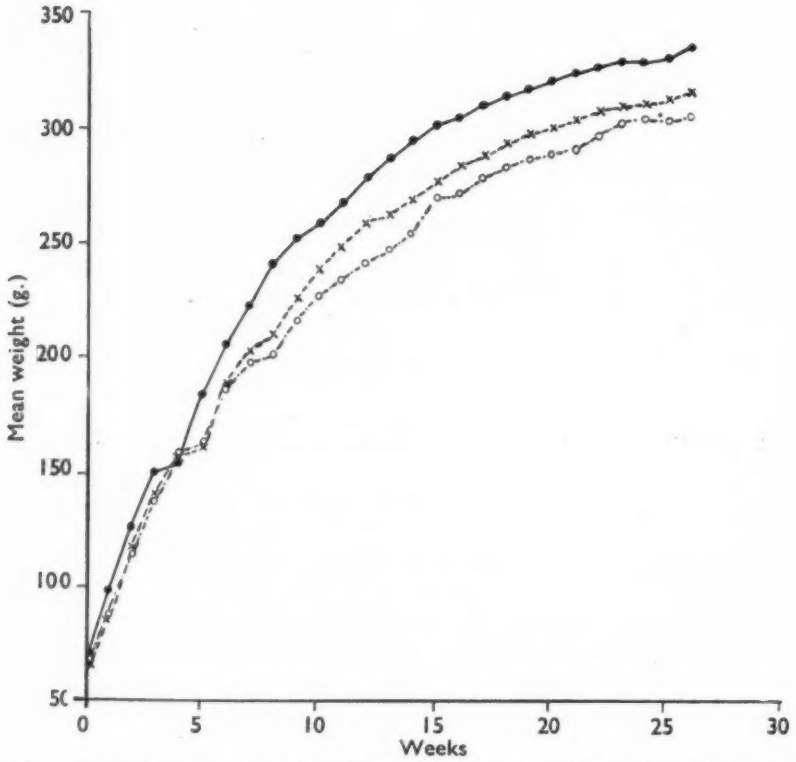

FIG. 5.—Growth-curves of rats fed on M.R.C. diet No. 41 with and without addition of 4-HIPA or aspirin. ●—●, no addition; ×– – ×, 0.2% 4-HIPA; O– . – O, 0.2% aspirin.

spleen, kidney, heart, lung, and testis of all rats in the chronic toxicity test showed no pathological effects attributable to 4-HIPA or aspirin. The rat mentioned above, which lost weight and showed abdominal enlargement, had a glandular hyperplasia resembling an adenocarcinoma in the large intestine. This was not attributed to the drug treatment.

Antagonism and Synergism

In view of the possible clinical use of 4-HIPA and because light might be thrown on its mode of action, we examined the effects of administering to rats a number of established drugs jointly with 4-HIPA.

Nalorphine. — Nalorphine was administered subcutaneously 20 min. before an intraperitoneal dose of 15 mg./kg. codeine or 450 mg./kg. 4-HIPA, the dose-ratio of nalorphine to each analgesic being 1 to 2. Nalorphine completely suppressed the analgesic effect of codeine: but it had no effect on that of 4-HIPA.

Other Analgesics. — Administering codeine subcutaneously and 4-HIPA intraperitoneally, we determined in the same experiment the analgesic potencies of the drugs given alone and together. The pooled results of three experiments are expressed graphically by the method described by Gaddum (1953) in Fig. 6(a), where each point was obtained with 40 rats. In this figure the ED50 isobol falls below the line of addition, indicating potentiation. Similar experiments in which we determined acute toxicity are expressed in Fig. 6(b), which indicates that toxicities were approximately additive.

Analgesic experiments of a similar type with 2-HIPA and codeine, in a total of 220 rats, also showed potentiation. In further experiments, morphine, pethidine, and phenacetin failed to potentiate, but showed an additive effect with 4-HIPA.

Hypnotics.—Unlike 4-HIPA, the hypnotic drugs studied caused the rat to lose its righting reflex; but doses ineffective in this way did not produce analgesia in our test. In experiments on synergism, we determined the effect of a constant dose

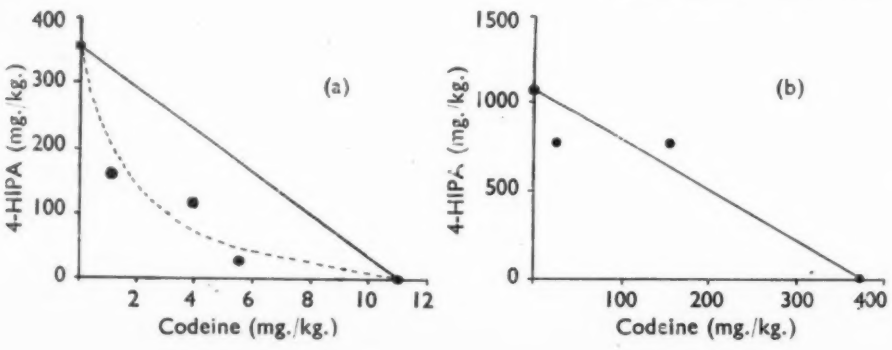

FIG. 6.—Synergism of 4-HIPA and codeine. 4-HIPA administered intraperitoneally, codeine subcutaneously, to young rats. (a) Analgesic ED50's; (b) LD50's.

of one drug on the potency of the other. Table III illustrates the enhancement of the analgesic activity of 4-HIPA by 100 mg./kg. methylpentynol and Table IV the enhancement of the hypnotic activity of methylpentynol by 450 mg./kg. 4-HIPA.

TABLE III

ENHANCEMENT OF ANALGESIC ACTIVITY OF 4-HIPA BY METHYLPENTYNOL

Both drugs were given intraperitoneally

Methylpentynol (mg./kg.)	4-HIPA (mg. kg.)	Proportion of Rats Showing Analgesia	ED50 (mg./kg.)
100	—	0/20	—
—	450	12/20	
—	150	7/20	363
100	150	14/20	
100	50	7/20	79

TABLE IV

ENHANCEMENT OF HYPNOTIC ACTIVITY OF METHYLPENTYNOL BY 4-HIPA

Both drugs were given intraperitoneally

Methylpentynol (mg./kg.)	4-HIPA (mg. kg.)	Proportion of Rats Losing Righting Reflex	ED50 (mg./kg.)
—	900	0/10	—
300	—	6/10	
225	—	2/10	282
200	—	0/10	
200	450	6/10	
150	450	4/10	174

In acute intraperitoneal toxicity tests, the LD50 of methylpentynol alone was 537 mg./kg. and that of methylpentynol in the presence of an equal dose of 4-HIPA was 555 mg./kg. These figures give no evidence of synergism in toxicity between the two drugs.

Experiments similar to those with methylpentynol were performed with thiopentone or pentobarbitone and 4-HIPA. 10 mg./kg. of either barbiturate alone had no analgesic action, but more than doubled the analgesic potency of 4-HIPA. Conversely, 450 mg./kg. 4-HIPA raised the hypnotic potency of thiopentone by 1.7 and of pentobarbitone by 1.8 times.

In toxicity tests, the LD50 of pentobarbitone alone was 86 mg./kg. and in the presence of 13 times its weight of 4-HIPA was 58 mg./kg. There thus appears to be slight synergism in toxicity between the barbiturate and 4-HIPA.

DISCUSSION

The failure of nalorphine to antagonize 4-HIPA strengthens the argument that this compound does not belong to the morphine type of analgesic. Since both 2- and 4-HIPA exhibit antipyretic properties, we may place them in the group of analgesic-antipyretics, of which aspirin is the most widely used. Absence of drowsiness in rats at analgesic doses, however, marks a difference of 2- and 4-HIPA from salicylamide and phenacetin.

In rats, the acute toxicity of 4-HIPA was less than half and the chronic toxicity no higher than that of aspirin. Aspirin failed to show analgesic action at the highest tolerated dose given (300 mg./kg.); on the other hand, 300 mg./kg. 4-HIPA produced analgesia in about half the animals treated and higher tolerated doses were effective in nearly all rats. The acute toxicity of 4-HIPA was rather lower than that of 2-HIPA. One of our colleagues, who was strongly hypersensitive to aspirin, did not show any reaction to 4-HIPA. These facts pointed to the clinical trial of 4-HIPA as an alternative to aspirin.

Although admixture with sodium bicarbonate lowered the *in vivo* activity of 4-HIPA, the analgesic effect of the free acid in the rat was clearly specific and not due to acidity, since buffer solutions at the same *p*H were used as controls. Moreover, related acids, such as *o*-, *m*-, and *p*-phthalic and 5-hydroxy*iso*phthalic, showed much less activity. It also seems unlikely that the low activity of the 4-HIPA–$NaHCO_3$ mixture was due to failure of absorption from the peritoneal cavity, since a similar mixture was of low activity intravenously. Robinson, Fehr, and Fitzgerald (1956) have found that the rate of urinary excretion of 4-HIPA after giving the sodium salt intraperitoneally is much higher than after the free acid. This suggests that the effect may result from an increased rate of clearance of the sodium salt.

After 8 intraperitoneal doses of 4-HIPA or aspirin, rats became insensitive to both codeine and 4-HIPA administered by the same route. This effect may be attributable to the peritoneal hypertrophy, which was seen *post mortem*.

Most rats receiving 4-HIPA by the oral route failed to show an analgesic response; but an oral dose enhanced the effect of a simultaneous intraperitoneal dose. This is consistent with the finding of Robinson *et al.* (1956) that rats absorbed 4-HIPA much less completely from the gut than from the peritoneal cavity.

The main biological actions of 2- and 4-HIPA were expected from their structural similarity to salicylic acid. It is therefore surprising that, although both 2- and 4-hydroxy*iso*phthalic acids have been known for over 80 years, these properties do not seem to have been reported previously. We found similar properties also, though to a lesser

degree, in the unsubstituted phthalic acids (Chesher *et al.*, 1955) and related compounds.*

Summary

1. In rabbits treated with *Proteus* pyrogen, 4-hydroxy*iso*phthalic acid (4-HIPA), given by the intraperitoneal route, exhibited antipyretic activity at least as great as that of aspirin. 2-Hydroxy-*iso*phthalic acid (2-HIPA) also showed antipyretic activity.

2. Using a tail-pressure method in rats, 2- and 4-HIPA, administered intraperitoneally, showed analgesic action unaccompanied by loss of righting reflex, drowsiness or other visible side-effect. 2- and 4-HIPA were of about equal potency, which was greater than that of aspirin, but less than those of salicylamide and codeine.

3. In acute experiments in rats, 2-HIPA was weight for weight less toxic than codeine, aspirin or salicylamide: 4-HIPA was less toxic than 2-HIPA.

4. The subacute and chronic toxicity of 4-HIPA in mice and rats was of the same order as that of aspirin. When 0.2% 4-HIPA was administered for 6 months in the diet to young rats, no toxic effects were seen except a very slight depression of growth-rate.

5. Nalorphine did not antagonize the analgesic activity of 4-HIPA.

6. Codeine potentiated the analgesic activities of 2- and 4-HIPA. Methylpentynol, pentobarbitone and thiopentone enhanced the analgesic activity of 4-HIPA, and 4-HIPA enhanced the hypnotic action of these three drugs.

**Note added in proof.* The analgesic activities of some related acids in comparison with 4-HIPA (=1.0) were: *o*-phthalic, 0.4; *m*-phthalic (*iso*phthalic), 0.4; *p*-phthalic (*tere*phthalic), 0.5; 5-hydroxy*iso*phthalic, 0.3; 4,5-dihydroxy*iso*-phthalic, 0.9; hydroxy*tere*phthalic, 0.7; 2,3-dihydroxy*tere*phthalic, 0.6; 2,5-dihydroxy*tere*-phthalic, 0.6; trimesic, 0.5; hydroxytrimesic, 0.5; 2,4,6-trihydroxybenzoic (phloroglucinol carboxylic), 0.5. Some of the compounds were kindly supplied by Drs. J. Idris Jones, N. R. Campbell, J. H. Hunt and E. P. Taylor.

We are indebted to Dr. J. Idris Jones, of the Chemical Research Laboratory, Teddington, for supplies of 2- and 4-HIPA; to Dr. D. N. Gupta, of University College Hospital Medical School, for histological examinations; to Dr. E. P. Taylor for 5-hydroxy*iso*-phthalic acid, and for the calcium salt of 4-HIPA; to Mr. H. W. Martin for helpful discussions; to Miss P. Harknett for technical assistance; and to the directors of Allen and Hanburys Ltd. for permission to publish this work.

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ANALGESIC ACTION IN A SERIES OF *N*-SUBSTITUTED ETHYL 4-PHENYLPIPERIDINE-4-CARBOXYLATES

BY

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Following the synthesis of pethidine by Eisleb and Schaumann (1939), a large number of its analogues have been tested for analgesic potency. The present series, synthesized by R. F. Anderson, P. M. Frearson, and E. S. Stern in the Research Department of J. F. Macfarlan and Co. Ltd., comprises derivatives in which the *N*-methyl group of pethidine has been replaced by a tertiary amino alkyl group. The substances tested are listed in Table I.

METHOD

Analgesia was measured in rats by the pressure method of Green and Young (1951), using apparatus different in some ways from that described. The pressure was measured with a mercury manometer, the bore of which was 3 mm. It was transmitted to the tail by a strip of wood (12×4×5 mm.) which was cemented to the plunger head of the large vertical syringe and applied to the tail at right angles about 1 cm. from the tip. The small horizontal syringe was operated by hand and was fitted with a spring under compression between the barrel and plunger head, to ensure a rapid release of pressure. A narrow capillary tube with a valve in parallel was later introduced between the two syringes; this restricted the rate of increase of pressure without unduly affecting the rate of release. The syringes were of 20 ml. and of 2 ml. capacity and their cross-sectional areas (and thus the applied forces) were in the ratio 6.5:1. A smaller ratio required more effort from the operator, and a steady increase of pressure was more difficult to achieve. In practice, pressure was increased until the rat squeaked, when the height of the mercury column was noted and the pressure released.

Green and Young abandoned quantitative measurements of analgesia in favour of an all-or-none method. This may involve waste of information, and we persisted in using the technique quantitatively. The difficulty is that, with effective doses of an analgesic, some rats fail to respond to any pressure, and an arbitrary value has to be allotted to obtain a mean value for a group. To avoid this we have taken as an index of analgesia the ratio

$$\frac{\text{Pressure required to induce a squeak before injection}}{\text{Pressure required to induce a squeak after injection}}$$

If a rat did not squeak with a pressure four times the control figure the index was taken as 0, which, unlike infinity, can be included in a mean value for a group. The index was calculated from the pressure measured at 15, 30, and 45 min. after injection. The substances tested did not differ markedly in duration of action, and we therefore calculated the mean of these three values to give a single index for the response of each rat. The inclusion of a control pressure might be expected to render the index independent of the dimensions of the apparatus and of the size of the rat's tail. Green and Young (1951) showed that the variation of the control pressure between rats of the same age was no greater than between different trials on the same rat. On some occasions, therefore, we have used, as numerator in each index, the mean control pressure of the rats used on that occasion. At other times the individual control pressures have been used.

All substances tested were injected subcutaneously in 4 ml. saline/kg. body weight.

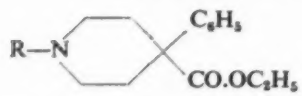
RESULTS

The first compound in the series, TA1 (Table I), was soon found to be considerably more potent than pethidine, and doses of 5 mg./kg. given to 50 g. rats frequently induced complete analgesia.

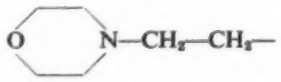
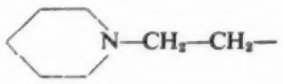
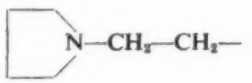
The use of rats on more than one ocasion did not reveal the development of tolerance, and the cross-over technique was used for a direct comparison of TA1 and pethidine. Two separate tests were carried out, each comprising a (2+2) dose, 4-way cross-over test in which 24 rats were used. In the first test the rats were divided into four groups of six (body weight evenly distributed, but otherwise at random), each group receiving in turn pethidine, 4 mg./kg. and 8 mg./kg., and TA1, 1.5 and 3 mg./kg. This test was so arranged that the groups received the four doses in a different order, and there was a 2- or 3-day interval between each dose. In the second test the doses were increased to 8 and 16 mg./kg. pethidine, and to 2.5 and 5 mg./kg. TA1; each rat was given these doses in a different order—using all the 24 possible ways—at 3-day intervals.

TABLE I

RELATIVE ANALGESIC ACTIVITIES OF ETHYL 4-PHENYL-PIPERIDINE-4-CARBOXYLATES



(In pethidine: R is CH_3)

TA No.	R	Salt	Relative Activity
1		2 HCl	100
2		2 „	<5
3		2 „	—
4		2 „	—
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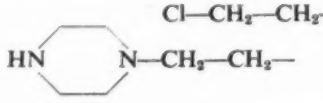
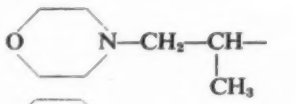
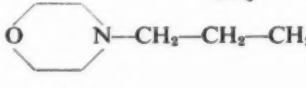
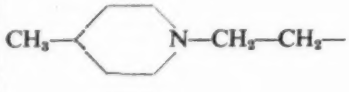
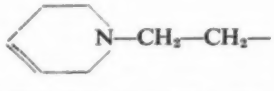
6		3 „	—
7		2 „	40
8		2 „	15
9		2 „	—
10		2 „	—
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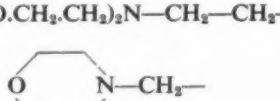
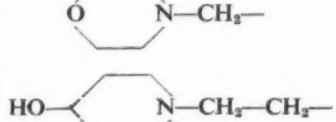
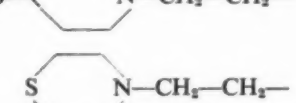
14		2 „	?5–10
16		2 „	—
18		2 „	33

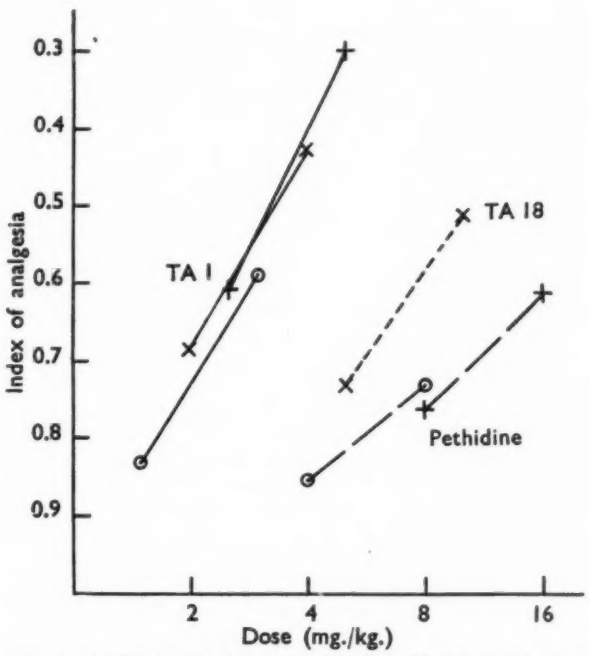


FIG. 1.—Values for the index of analgesia plotted against dose of drug. Each point is the mean from 24 rats obtained in a (2 + 2) dose 4-way cross-over test. All points from the same test are indicated by the same symbol. Points from the first test comparing TA1 and pethidine are marked ⊙; points from the second test of TA1 and pethidine are marked +; and those from the test comparing TA1 and TA18 are marked ×. The pairs of points for the two doses of a drug used in any one test are joined by straight lines.

In the first test the analysis of variance showed the log dose response slopes to be significantly different, so that the potency ratio (TA1 to pethidine), calculated from this test to be 4.8, has no absolute value. This was confirmed by the second test, where larger doses gave the higher potency ratio of 6.5 (fiducial limits 4.51 and 9.47, $P=0.95$). Here the difference in slopes was also apparent (see Fig. 1), but owing to the greater error variance (Table II) the difference was not quite significant at the 5% level.

Most of the other compounds in the series were without appreciable activity. TA3, TA6, TA13, and TA16 produced no effect when tested in doses of at least 50 mg./kg. TA2 had some effect: the mean index in a group of six rats given 80 mg./kg. was 0.62. TA5, 100 mg./kg., produced a definite response (the mean index for six rats was 0.48).

Solubility limited the doses of TA4, TA9, and TA10, and no appreciable analgesia was seen. For six rats given 22.5 mg./kg. TA4 the mean index was 0.84; for five rats given 25 mg./kg. TA9 it was 0.97; for five rats given 37.5 mg./kg. TA10 it was 0.95. TA14 was only available in very small quantity; 30 mg./kg. was injected in four rats and

TABLE II

COMPARISON OF TA1 AND PETHIDINE: ANALYSES OF VARIANCE

Source of Variation	d f.	Sum of Squares	Variance	Variance Ratio	P
First Comparison					
Between drugs	1	0·4874	0·4874	32·9	<0·1%
Regression ..	1	0·6767	0·6767	45·7	<0·1%
Deviation from parallelism ..	1	0·1395	0·1395	9·4	<1·0%
Between days ..	3	0 1399	0·0466	3·15	<5%
,, rats ..	23	0·9332	0·0406	2·74	<1%
Error	64*	0·9472	0·0148		
Total ..	93*	3·3239			
Second Comparison					
Between drugs	1	1·3090	1·3090	30·4	<0·1%
Regression ..	1	1·2308	1·2308	28·6	<0·1%
Deviation from parallelism ..	1	0·1528	0·1528	3·5	>5%
Between days ..	3	0 0504	0·0168	0·391	—
,, rats ..	23	0·9013	0·0392	0·912	—
Error	66	2 8358	0·0430		
Total ..	95	6·4801			

* Observations could not be made on one rat after the first two days of the first test. Values calculated to avoid biasing the means for the appropriate group on each of the two remaining days were inserted in the results. The degrees of freedom associated with error were consequently reduced to 64 in this test.

there was probably some effect (mean index 0.73). TA7 and TA8 were both found to be effective analgesics. The relative activities shown in Table I are based on a test in which TA7 (12.5 mg./kg.), TA8 (25 mg./kg.), and TA1 (2 mg./kg. and 4 mg./kg.) were given to four groups of five rats. The test was repeated 7 days later, the drugs being interchanged so that each dose was given to ten rats in all.

So far the only substances in this series to show appreciable activity contained the morpholino ring, and the oxygen atom appeared essential. It therefore became of considerable interest to obtain and test the analogous compound containing sulphur in place of oxygen. This compound, TA18, was found to be quite active and was compared with TA1, in a (2+2) dose 4-way cross-over test similar to the second of the tests in which TA1 and pethidine were compared. In this test the slopes for TA1 and TA18 were parallel (the appropriate variance was actually less than the error variance) and TA1 was 3.03 (limits 2.46 and 3.74, $P=0.95$) times as active as TA18.

Pethidine, like morphine, is antagonized by nalorphine, and we found that 2 mg./kg. of nalorphine was effective in antagonizing the analgesic action of 4 mg./kg. of TA1 when the two drugs were given simultaneously; however, this dose of nalorphine was much less effective against 10 mg./kg. of TA1; 24 mg./kg. of nalorphine exerted an analgesic action of its own (Table III).

TABLE III

NALORPHINE ANTAGONISM TO THE ANALGESIC ACTION OF TA1

Group of 6 Rats	Dose mg./kg.		Mean Index of Analgesia
	Nalorphine	TA1	
I	—	4	0·18
II	2	4	0·83
III	12	—	1·01
I	24	—	0·67
II	—	10	0·19
III	2	10	0·58

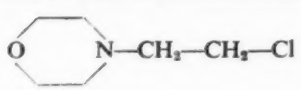
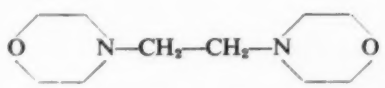
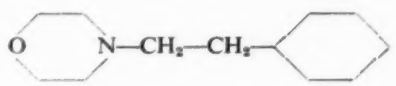
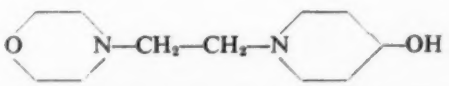
The tests shown in the last 3 rows were done 7 days after the first tests using the same 3 groups of rats.

The substituent groups in this series of compounds are introduced into pethidine in a similar position to that in which the allyl group is introduced into the morphine molecule to give nalorphine. We therefore investigated the possibility of antagonism of TA1 by TA2 or TA3. Nine rats were injected with 5 mg./kg. TA1. In three rats this was preceded by 50 mg./kg. TA2; analgesia was complete, the mean index being 0. In another three rats the dose of TA1 was preceded by 50 mg./kg. TA3; the index was 0.04. The remaining three rats were given saline before the injection of TA1, and here the index was 0.17. The effect of TA2 or TA3 with TA1 is, if anything, additive rather than antagonistic.

The importance of the morpholino ring when attached to the pethidine nucleus lent some interest to several simpler compounds containing this ring (see Table IV), but it was not surprising to find that they were not effective. In a dose of 100 mg./kg., TA11 did raise the pain threshold, but 5 of the 6 rats given this dose were dead the day

TABLE IV

SIMPLE DERIVATIVES OF MORPHOLINE TESTED

TA11	
TA15	
TA17	
TA19	

after the test. Smaller doses did not produce an analgesic effect, but induced a permanent disability, causing the rats to "waltz." This effect has previously been observed by Goldin, Noe, Landing, Shapiro, and Goldberg (1948) with several compounds, including TA11. The other compounds of this group, TA15, TA17, and TA19, showed no analgesic activity in doses of 100 mg./kg.

Toxicity of Morpholinoethylnorpethidine Hydrochloride (TA1)

Acute.—Unlike pethidine, TA1 did not induce convulsions in mice, but the Straub tail reaction was frequently seen. There was some irritability, followed by prostration. Death occurred after a period of marked respiratory depression. The intraperitoneal LD50 in 10–20 g. mice was 118 mg./kg. The toxicity increased sharply with dose, and the fiducial limits for this estimate of LD50 were 113 and 123 mg./kg. In heavier mice the toxic dose, expressed in mg./kg., was somewhat lower. Duguid and Heathcote (1940) and Gruber, Hart, and Gruber (1941) give intraperitoneal LD50's for pethidine of 125 and 147 mg./kg. A small number of mice were injected subcutaneously with doses up to 600 mg./kg. of TA1 without any deaths.

In rats injected subcutaneously the individual lethal doses varied from 50 to 200 mg./kg. The LD50 was between 70 and 100 mg./kg. (In our experience the subcutaneous LD50 of pethidine is about 200 mg./kg., the figure quoted by Barlow and Lewis, 1951.)

Subacute.—Three groups of 10 rats were weighed and given daily subcutaneous injections over 11 days. One group received TA1, 20 mg./kg., the second pethidine, 60 mg./kg., and the third group was injected with saline only. One rat in the TA1 group died on the 10th day, while only four of the rats receiving pethidine survived to the end of the test. The rats were weighed and killed on the 12th day. The mean weight gain of the surviving rats in each group was 26.7 g. with TA1, 26 g. with pethidine, and 26 g. in the controls. The liver, kidneys, spleen, heart, lungs, and brain of the surviving rats were examined histologically. No pathological changes were observed in these tissues. The rat in the TA1 group which died on the 10th day of the test was also examined and showed the following pathological changes in sections of lung tissue: alveoli oedematous and engorged with blood; gross generalized congestion; diffuse haemorrhages; haemorrhagic fluid in bronchioles. No pathological changes were noted in the liver, kidneys, spleen, or heart.

Discussion

We have found the pressure method of Green and Young to be a useful technique for observing the effects of analgesic drugs. The "index of analgesia" described in the section on method is a convenient extension of the original procedure.

Tests on small groups of rats soon showed that morpholinoethylnorpethidine was superior to any of the other compounds in the series, and relatively little effort was expended on these others. It was rather surprising that the presence of oxygen or sulphur in the heterocyclic ring should be so important for analgesic action. The compound with the morpholino ring is 3–7 times more potent than the parent compound, pethidine, whereas compounds without the oxygen (or sulphur) atom, but otherwise closely similar, are practically inactive despite the fact that nearly all the pethidine structure remains intact.

If the chain of carbon atoms linking the nitrogen atoms of the two rings in TA1 is lengthened (TA8) or shortened (TA14) there is a considerable reduction of activity. Branching also reduces activity (TA7), though not sharply. TA4 comprises essentially two molecules of pethidine joined together, but has little or no analgesic activity.

The use of a cross-over technique for comparing analgesics is not usual. The possibility of tolerance developing has presumably kept others from using this procedure although tolerance would not necessarily invalidate a symmetrical assay. In the present tests no evidence of tolerance was observed and the practical advantages would seem to outweigh theoretical disadvantages. In the first of the two cross-over tests in which TA1 and pethidine were compared the design was poor. The effects of the different doses were confounded with possible effects arising from the animals being housed in 4 separate cages: in addition all animals given a particular dose on the 2nd, 3rd, and 4th days of the test had a common dose history which was different from the histories of the rats given the other doses. This assay could therefore be misleading; but in fact the results agreed well with the second test, in which these faults were eliminated. The second design had the additional advantage that the doses were injected in irregular order down the list of rats; this eliminated the possibility of systematic effects due to the order of injecting and testing, and also made the actual testing of threshold more objective, since the observer did not remember at the time of testing

which dose each rat had received. This made it unnecessary to take special precautions to ensure that the observations were unbiased, which otherwise would have been very desirable because of the occasional difficulty in deciding the precise end-point.

When different substances are compared in a biological test it frequently happens that the dose-effect lines are not parallel. The errors of such tests are large, so that differences in slope are usually not significant; but with pethidine and morpholinoethylnorpethidine the difference in slope is sufficient to make it reasonably certain that the difference is real. This has the immediate disadvantage that it is not possible to state that TA1 is "R" times more potent than pethidine, but it also indicates that TA1 may be qualitatively as well as quantitatively a better analgesic than pethidine. It is generally believed that some of the analgesics used clinically produce a maximal effect larger than that produced by others, and it is possible that this difference is analogous to the difference of slope between TA1 and pethidine which we have observed in rats. There is thus some reason for supposing that TA1 may be more useful than pethidine in the treatment of severe pain. In rats the difference between the drugs is such that doubling a dose of TA1 is as effective as quadrupling the equi-active dose of pethidine.

The few toxicity tests that we have done provide some ground for supposing that the increase in potency seen with TA1 is not accompanied by as great an increase in toxicity. The tests in mice, though favourable to TA1, are probably not of much value, since the absence of marked convulsions with TA1 makes close comparison with pethidine irrelevant. In rats death follows a period of depression with both substances, and a comparison of toxicity has some value. The rather wide range of the individual lethal doses would require the use of a large number of animals to give a precise comparison; but our results suggest that TA1 is not three times more toxic than pethidine—and this is about the minimum value of the ratio of analgesic activity. The subacute toxicity test confirmed that TA1 is less than three times as toxic as pethidine, since, with this ratio of doses, pethidine killed 6 of 10 rats, and TA1 killed only 1 of 10, over the period of eleven days.

Summary

1. A series of compounds in which the *N* methyl group of pethidine is replaced by a tertiary amino alkyl group has been tested for analgesic activity in rats.

2. One of these, morpholinoethylnorpethidine, is considerably more potent than pethidine and its toxicity is not correspondingly high.

We wish to thank Dr. R. F. Ogilvie, Department of Pathology, Edinburgh University, to whom we are indebted for examining the sections, and Dr. A. H. B. Masson, who tested compounds TA13 and TA14.

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ANALGESIC AND OTHER PROPERTIES OF MORPHOLINOETHYLNORPETHIDINE

BY

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Millar and Stephenson (1956) have investigated the analgesic action of a series of aminoalkyl derivatives of pethidine prepared by Anderson, Frearson, and Stern (1954, private communication). The most powerful of these compounds was morpholinoethylnorpethidine hydrochloride (TA1) with an analgesic activity of at least three times that of pethidine in rats. Further properties of this compound are reported here.

METHODS

The same procedures have been used to test effects on pain threshold, respiration, and rectal temperature as in the investigation of other analgesics in this laboratory (Green and Young, 1951; Green, 1953). The analgesic estimates in Table I were derived by quantal analysis of a test in which pain thresholds to heat and to pressure were measured 30 min. after subcutaneous injection of TA1 and morphine at each of three dose levels in groups of ten 3–4-week-old Wistar rats. Respiratory frequencies were determined at the same time as the pain threshold measurements. Effects on the cough reflex in anaesthetized cats and antagonism by nalorphine of the analgesic and respiratory depressant properties were determined by the methods used by Green and Ward (1955) and Green, Ruffell, and Walton (1954) respectively.

RESULTS

Analgesia.—TA1 was capable of causing as great an elevation of pain threshold as morphine in rats, and its relative potency by subcutaneous injection was intermediate between that of morphine and pethidine in this species (Table I). This is probably also true by subcutaneous injection in dogs, since 5 mg./kg. TA1 elevated the pain threshold by at least 100% and caused moderate narcosis in five of six dogs and since its intensity and duration of action were comparable with those previously found (Green, 1953) with 1–2 mg./kg. morphine sulphate or 20 mg./kg. pethidine hydrochloride. Argent (1955, private communication) has found that in man, too, the activity of the morpholino compound is greater than that of pethidine and less than that of morphine.

TABLE I

DOSES RAISING THE PAIN THRESHOLD TO HEAT AND TO PRESSURE BY AT LEAST 100% IN 50% OF RATS (ED50), AND RELATIVE ANALGESIC POTENCIES

The limits of error, $P=0.95$, are shown in parentheses

	ED50 (mg./kg.)		Relative Potency	
	Heat	Pressure	Heat	Pressure
TA1	5·2 (2·9–9·1)	2·6 (1·7–4·0)	0·58 (0·31–1·1)	0·54 (0·28–1·0)
Morphine sulphate	3·0 (2·3–4·0)	1·4 (0·86–2·2)	1·0	1·0
Pethidine hydrochloride			0·2*	0·27*

* Comparative estimates of Green and Young (1951).

In each of two cats 2.5 mg./kg. TA1 caused mydriasis and slight analgesia, but three hours later, when the cats had apparently fully recovered, a further dose of 10 mg./kg. caused marked excitement.

Toxicity and Respiratory Depression.—The acute intravenous LD50 of TA1 in mice was approximately 45 mg./kg. Unlike pethidine it did not cause violent convulsions. Respiratory depression seemed to be its main toxic action in this species and also in rats, rabbits, cats and dogs. Other signs in mice included such morphine-like effects as tail erection, analgesia, mydriasis, and bursts of hyperactivity.

The mean respiratory frequencies of groups of 10 rats 30 min. after subcutaneous injection are plotted against the dose of the analgesics in Fig. 1. Relative to its analgesic activity in this species, the respiratory depressant action of TA1 is not significantly different from that of morphine. The respiratory minute volume was depressed by at least 70%, largely by reduction of frequency, after 15 mg./kg. TA1 subcutaneously in each of

five rabbits. In normal dogs the frequency of breathing was slowed after 5 mg./kg. TA1 subcutaneously, and in a dog anaesthetized with pentobarbitone sodium (40 mg./kg.) the rate was reduced from 16/min. to 10/min., for several minutes after 1 mg./kg. intravenously. In each of two cats, 1 and 3 mg./kg. intravenously reduced the rate by about 30% and 50% respectively.

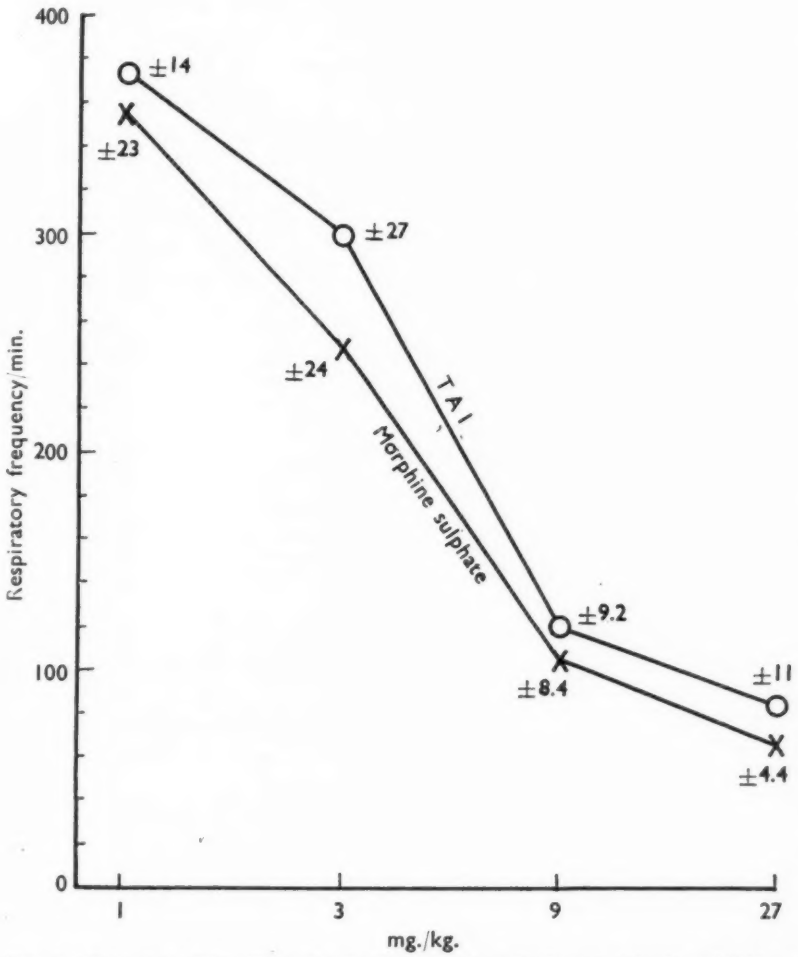

FIG. 1.—Frequency of breathing of rats 30 min. after subcutaneous injection of TA1 or morphine sulphate. Means for groups of ten rats and standard errors. Controls 376±18.

Antitussive Action.—Coughs caused by electrical stimulation of the superior laryngeal nerve were reduced in intensity by 0.3 mg./kg. and abolished by 1 mg./kg. TA1 in each of two anaesthetized cats; in one of these animals coughs caused by mechanical irritation or SO_2 were similarly affected. Pethidine (1–3 mg./kg.) and morphine (0.3–1.0 mg./kg.) have produced effects comparable with this (Green and Ward, 1955).

Temperature.—After 8 mg./kg. TA1 subcutaneously the fall in rectal temperature of 2 rabbits was slightly greater, but of shorter duration, than that after 4 mg./kg. morphine sulphate in 2 other rabbits.

Gastro-intestinal Effects.—Unlike morphine, but in common with many synthetic analgesics, TA1 was not emetic in dogs, but salivation and defaecation occurred in 4 of 6 dogs soon after the subcutaneous injection of 5 mg./kg. As with other morphine-like analgesics TA1 inhibited the peristaltic reflex of isolated guinea-pig ileum, the inhibition being partial at 3×10^{-7} and complete at 10^{-6}. Concentrations of 10^{-6} did not prevent acetylcholine (5×10^{-7}) or histamine (5×10^{-7}) from causing maximal contractions of isolated guinea-pig ileum.

Cardiovascular Effects.—Slight bradycardia and a fall of 30 mm. Hg in the mean carotid blood pressure occurred after intravenous injection of 1 mg./kg. TA1 in a dog anaesthetized with pentobarbitone sodium. A dose of 3 mg./kg. reduced the heart rate from 170/min. to 45/min. and lowered the mean carotid blood pressure by 40 mm. Hg. The bradycardia and the fall in blood pressure were abolished by atropine (0.1 mg./kg.), and so, as with morphine, these actions are presumably due to vagal excitation. After atropinization, doses of 10–40 mg./kg. TA1 reduced blood pressure without slowing the heart.

Action on the Pupil.—As with other morphine-like analgesics, TA1 caused mydriasis in mice and cats and myosis in dogs.

Antagonism by Nalorphine.—Nalorphine inhibited several of the morphine-like properties of TA1. Analgesia caused by large doses (25 mg./kg.) of TA1 in rats was very greatly reduced by as little as 1 mg./kg. nalorphine (Table II), and

the respiratory frequency in these animals was restored to normal. Respiratory minute-volume, depressed after 15 mg./kg. TA1 subcutaneously in rabbits, was restored to normal by 0.5 mg./kg. nalorphine intravenously in each of five animals. The suppression of the cough reflex and the reduction in respiratory frequency caused by 3 mg./kg.

TABLE II

ANTAGONISM OF THE ANALGESIC ACTION OF TA1 BY NALORPHINE HYDROCHLORIDE IN RATS

The pain thresholds were measured 30 min. after subcutaneous injection of the drugs

		Incidence of a 100% Rise in Threshold		ED50 of Nalorphine and Limits, $P=0.95$ (mg./kg.)	
TA1 (mg./kg.)	Nalorphine (mg./kg.)	Heat	Pressure	Heat	Pressure
25	0	10/10	10/10		
25	0·25	10/10	10/10	0·99	2·6
25	1·0	4/10	7/10	(0·57–1·7)	(1·2–5·3)
25	4·0	1/10	4/10		

TA1 in two anaesthetized cats were antagonized by 0.3 mg./kg. nalorphine. The morphine-like excitement caused by 10 mg./kg. TA1 in non-anaesthetized cats was also abolished by 2 mg./kg. nalorphine intraperitoneally. Similarly, whereas the peristaltic reflex of isolated guinea-pig ileum was, in the absence of nalorphine, abolished by TA1 at 10^{-6}, as much as 10^{-5} was required to cause inhibition in the presence of 10^{-7} nalorphine.

Chronic Toxicity.—Daily subcutaneous injection of 10 mg./kg. TA1 in newly weaned rats for 9 weeks caused only a very slight retardation of growth and no change in the histological appearance of the lungs, livers, spleens, or kidneys.

SUMMARY

1. Morpholinoethylnorpethidine is a powerful analgesic, and its activities in rats and dogs are intermediate between those of pethidine and morphine.

2. This new compound depressed respiration in several species ; in rats this action was as powerful, relative to its analgesic potency, as that of morphine. Its actions on the cough reflex in anaesthetized cats, on rectal temperature in rabbits, on pupil diameter in mice, cats and dogs, and on heart rate in dogs also resembled those of morphine. Similarly it abolished the peristaltic reflex of isolated guinea-pig ileum and caused defaecation in dogs. In cats it produced morphine-like excitement.

3. Its effects on pain threshold, respiration, and the cough and peristaltic reflexes were antagonized by nalorphine.

We are indebted to Dr. E. S. Stern, of J. F. Macfarlan & Co. Ltd., who supplied the morpholinoethylnorpethidine hydrochloride, and to Mrs. E. P. Penson and Mrs. I. A. Saunders, who carried out many of the pharmacological tests described.

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THE EFFECT OF 5-HYDROXYTRYPTAMINE ON THE EXCRETION OF WATER IN CONSCIOUS DOGS

BY

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(RECEIVED OCTOBER 12, 1955)

5-Hydroxytryptamine (5-HT) is present in a number of tissues, but its physiological role is still undetermined. Erspamer (1954a, b,) found that 5-HT was powerfully antidiuretic and that, in rats, the subcutaneous route of administration was the most effective, as little as 4 μg./kg. producing definite and fairly long-lasting antidiuresis in the most sensitive individuals. By means of clearance measurements he found that glomerular filtration rate (GFR) was reduced and that there might also be some reduction in renal plasma flow (RPF). He concluded that 5-HT antidiuresis was due to preferential constriction of the afferent glomerular arterioles and, further, that the posterior pituitary played no part in the antidiuresis. He believes that 5-HT is a hormone designed for the physiological regulation of renal function (1953). Since most work on 5-HT has been done on rats, and little on dogs (Erspamer and Ottolenghi, 1951 ; Sala and Castegnaro, 1953), and since it is important to determine whether 5-HT is a renal hormone, as Erspamer believes, or merely an antidiuretic substance, we have carried out a number of different studies on the dog.

METHODS

Observations were made on conscious bitches during water diuresis. The methods of collecting urine and inducing diuresis have previously been described (Abrahams and Pickford, 1954). 5-HT was administered in the form of the creatinine sulphate dissolved in 1.0 ml., or less, of 0.9% NaCl solution. All doses are calculated and given as weight of base/kg. body weight of dog. The drug was injected subcutaneously, intramuscularly, intra-arterially or intravenously. The intra-arterial injections were made either into the sinus-denervated carotid artery lying in a previously prepared van Leersum loop, or into the aorta through a catheter inserted, under local anaesthesia, *via* the femoral artery. The catheter was inserted to a measured length immediately before giving the experimental dose of water. The position of its tip was ascertained later by reintroducing the catheter when the animal was killed. When intra-aortic injections were made the substance to be injected was dissolved in a volume of 0.9% NaCl solution less than that of the dead space of the catheter, namely, 0.8 ml. The test solution was injected into the catheter and then washed into the circulation with 1 or 2 ml. 0.9% NaCl solution.

Some observations were made on the renal clearances of diodone and creatinine before and after the intravenous injection of 5-HT. The methods used have already been described (Pickford and Ritchie, 1945). They avoid interference with the dog except for the collection of blood samples, and provide a period of 20 to 25 min. at the plateau of diuresis when the plasma concentrations of diodone and creatinine remain relatively constant. If antidiuresis occurs the period of steady plasma concentration is prolonged.

When required, blood pressure was measured in the conscious dog through a side-arm of the aortic catheter, and recorded by means of a mercury manometer.

RESULTS

Subcutaneous and Intramuscular Administration of 5-HT.—Only 4 observations were made on the effect on water diuresis of subcutaneously administered 5-HT. One bitch (Angela) received 22 μg./kg. 36 min. after the ingestion of water. The course of the diuresis thereafter was not noticeably different from normal. It was, however, possible that there had not been sufficient time for the drug to be absorbed and produce its effect. Therefore, in another animal (Darkie) 5-HT was given at the time of water administration. On one day the dose was 32 μg./kg. and on 2 others 74 μg./kg. subcutaneously. On no occasion did antidiuresis occur (Fig. 1).

Two animals were given 13.5 and 36 μg./kg. 5-HT intramuscularly and showed no hint of antidiuresis. On the other hand, 32 μg./kg. given intramuscularly to Darkie caused on this occasion an immediate and marked antidiuresis. The short latency of the response (1.0 min.) makes it probable that this positive response of Darkie to an

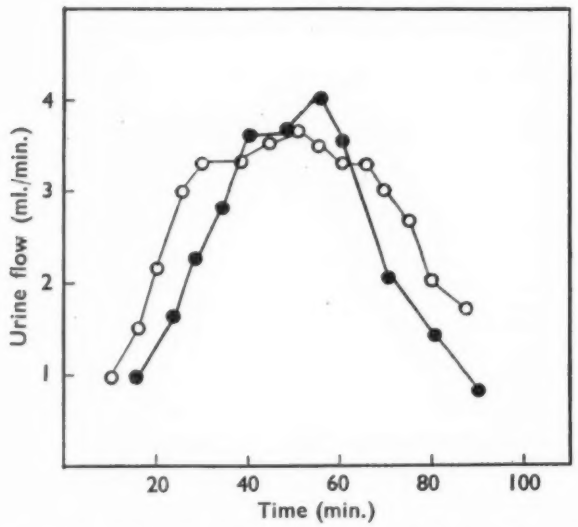

FIG. 1.—Record of water diuresis (Darkie). 300 ml. water by mouth at zero time. ●——● normal water diuresis. O——O, water diuresis when 74 μg./kg. 5-HT was given subcutaneously at zero time. In all graphs, ordinate is rate of urine flow in ml./min., and abscissa is time in min.

intramuscular injection when this animal gave consistently negative responses to subcutaneous injections was coincidental.

Administration of 5-HT into the Malleolar Vein.—A single injection of 5-HT given into the malleolar vein during water diuresis caused antidiuresis to a variable extent. The doses tested ranged from 0.6 μg./kg. to 84 μg./kg. Since the effect in any one dog varied from time to time (Fig. 2) despite apparently constant conditions, which included the time of day and the anoestrous condition of the animal, it was difficult to determine the minimum effective dose. Some dogs showed more variability of response than others. It was, however, clear that, in 11 normal dogs, true antidiuresis, as contrasted with momentary cessation of flow due to ureteric spasm (Abrahams and Pickford, 1956a), was not regularly seen with doses under 10 μg./kg. (Table I). Only one animal (Smokey) showed a sensitivity greater than this, and at post mortem this dog was found to have only the left kidney. On the right there was nothing but a fibrous cord in place of the ureter. The one kidney was large, dimpled on the surface, and showed, on histological examination, many areas of considerable fibrosis. In life there had been nothing to suggest that this dog was congenitally abnormal. This animal was one of the first on which observations were made, and unfortunately, before it was killed, information was given to Professor Erspamer that, in dogs, the sensitivity to intravenously administered 5-HT ranged from 4 to 20 μg./kg. Later experience amends these figures to 10–20 μg./kg.

TABLE I

MINIMUM INDIVIDUAL EFFECTIVE ANTIDIURETIC DOSES OF 5-HT WHEN INJECTION WAS MADE INTO THE MALLEOLAR VEIN

No. of Observations	Dog	Dose 5-HT μg./kg.	No. of Observations	Dog	Dose 5-HT μg. kg.
15	Frisk	10	6	Bouncer	14
2	Topsy	18·5	4	Darkie	16
3	Sheila	20	8	Angela	21·8
5	Jess	12·7	5	Kit	9·4
4	Chris	13·5	10	Judy	10
4	Lady[1]	18·5	5	Thisbe	13
6	Smokey[2]	4·0			

[1] With diabetes insipidus. [2] Congenital absence of right kidney.

Some observations were made on one dog at a time when it had a prolapsed uterus. It could be seen that, when antidiuresis occurred in response to an injection of 5-HT, there was a perceptible paling of the vaginal mucous membrane, lasting about 5 min.; when antidiuresis did not occur there was no pallor. Furthermore, all dogs which subsequently showed antidiuresis sighed, panted, or

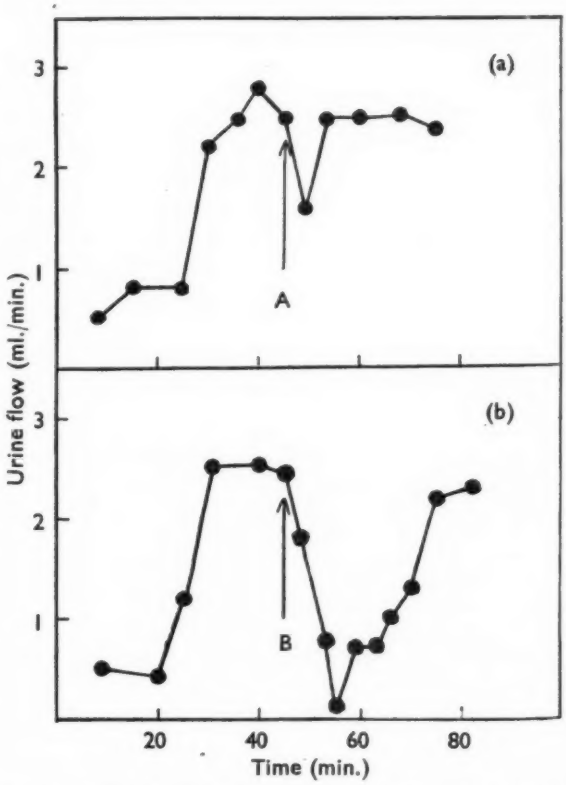

FIG. 2.—Record of water diuresis (Judy). 250 ml. water at zero time. In (a), at A, 34.8 μg./kg. 5-HT. In (b), at B, 17.4 μg./kg. 5-HT. Injections made into malleolar vein on both occasions.

yawned some seconds after the injection of 5-HT. If this respiratory response was minimal or absent antidiuresis did not occur.

Administration of 5-HT into the Carotid Artery.—Intracarotid injections of 5-HT were given 4 times in the following doses: 8.6 μg./kg., 9.2 μg./kg., 9.4 μg./kg., and 18.8 μg./kg. The results were variable, but it was clear that the effect of 5-HT was no greater nor was the minimum effective dose smaller when the drug was given into the common carotid instead of the malleolar vein (Fig. 3). These findings agree with those of others, who state that the action of 5-HT is not a central one (Erspamer, 1954a). With regard to the respiratory effects, these were no greater, and perhaps less, on intracarotid than on intravenous injection. This agrees with the findings of Douglas and Toh (1953) and of Schneider and Yonkman (1954).

Renal Clearances Following the Administration of 5-HT.—Renal clearances of diodine and creatinine were measured 15 times on 5 dogs before and after the injection into the malleolar vein of doses of 5-HT ranging from 9.2 to 18.5 μg./kg. The responses varied considerably, antidiuresis being observed in 9 of the 15 experiments. In the 9 experiments in which antidiuresis occurred, its degree varied from one or more transitory depressions to a prolonged inhibition of urine flow

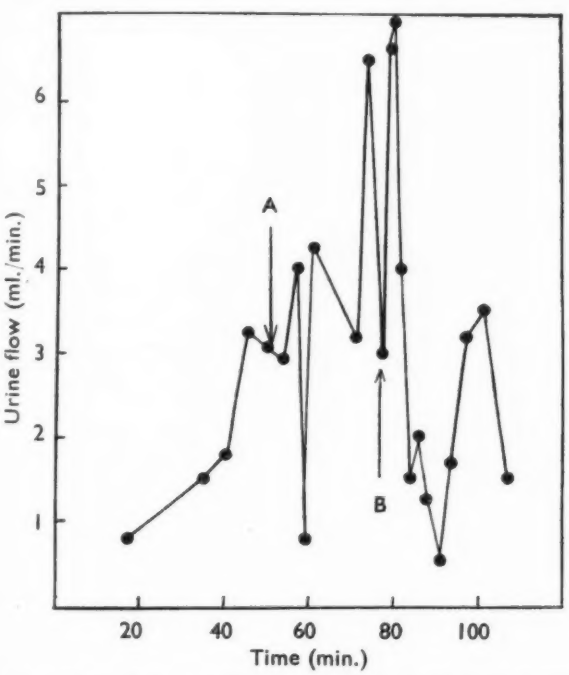

FIG. 3.—Record of water diuresis (Angela). 300 ml. water at zero time. At A, 18.8 μg./kg. 5-HT into the left common carotid artery. At B, 18.8 μg./kg. 5-HT into the malleolar vein.

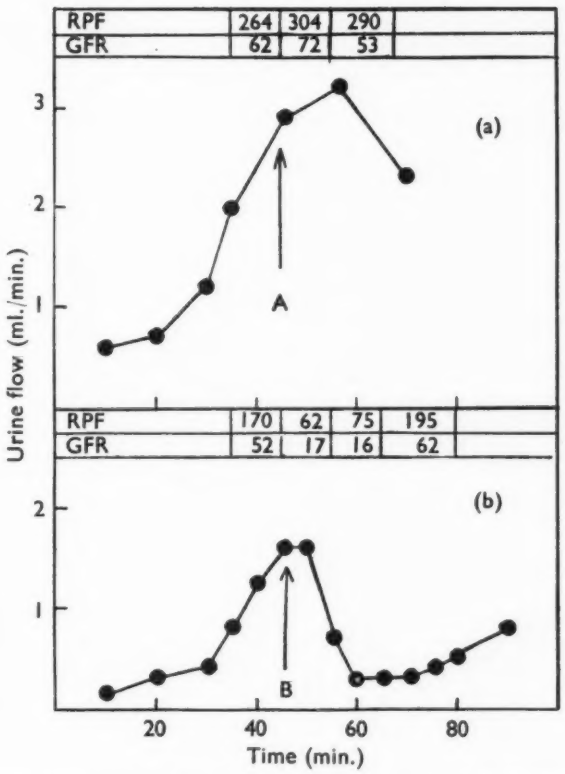

FIG. 4.—Record of water diuresis and renal clearances in ml./min. of diodone (RPF) and creatinine (GFR) in 2 dogs before and after injection into the malleolar vein of 5-HT. (a) Bouncer; at A, 10.2 μg./kg. (b) Judy; at B, 15 μg./kg.

(Fig. 4). When depression was transitory both clearances rose and fell roughly to the same extent and in parallel with the urine flow, suggesting a purely vascular origin for the alterations in renal activity. When there was prolonged inhibition of urine flow, both clearances fell in 7 out of 9 experiments; once the GFR alone and once the RPF alone decreased. After the initial depression which lasted for 20 to 25 min. the RFP rose towards or above the preinjection level, even though the urine flow was still inhibited. On three occasions the GFR also increased (Fig. 4).

When urine flow was not inhibited by the administration of 5-HT both clearance values rose to a greater or lesser extent (Fig. 4).

Thus 5-HT, in contrast to posterior pituitary lobe extract, is uncertain and variable in its action on dog kidney unless given in fairly large doses. The initial phase of 5-HT antidiuresis seems to depend on decreased plasma flow and glomerular filtration rate, the latter especially tending to persist. The fact that antidiuresis persisted after recovery of both clearances suggested that the preliminary reduction of blood supply to the kidney

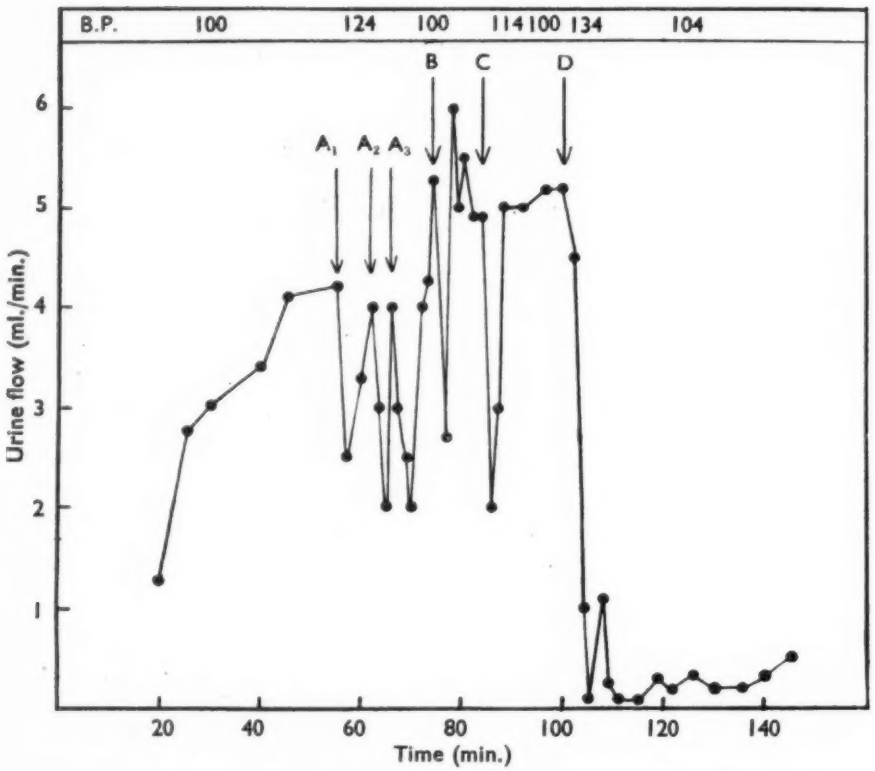

FIG. 5.—Record of water diuresis (Thisbe). 300 ml. water at zero time. A_1, A_2, and A_3, three separate intra-aortic injections of 10 μg. adrenaline. B, 8 μg./kg. 5-HT intra-aortically. C, 40 μg./kg. 5-HT intra-aortically. D, 20 μg./kg. 5-HT into the cubital vein. At top, blood pressure in mm. Hg.

gave rise to some other and more long-lasting reaction (Verney and Vogt, 1938, 1943), possibly reflex. Therefore, the reaction of the kidney to 5-HT injected into either the aorta or the cubital vein was examined. By this means information might be obtained about the origin of the vascular changes occurring in the kidney, i.e., whether the kidney shows a particular sensitivity to the drug, or whether it simply responds to a change in vascular conditions elsewhere in the body.

Administration of 5-HT into the Aorta.—The minor operation of aortic catheterization did not usually delay the onset of water diuresis. In five out of six experiments there was no difference from the normal pattern of diuresis ; only once was its onset delayed by 30 min. In all instances the catheter tip was one or more inches above the origin of the right renal artery. Control observations showed that the injection of 2 ml. saline alone into the aorta had no effect on the rate of urine flow.

One animal (Jess) received an intra-aortic injection of 22 μg./kg. 5-HT. Three min. after this dose the urine flow fell from its previous rate of 6 ml./min. to 5.3 ml./min., and 3 min. later was again 6 ml./min. This was the only noticeable response. Respiration was in no way disturbed. Owing to a clot in the catheter it was not possible to repeat the injection. The negative result could have occurred because the 5-HT was swept past the renal arteries and did not reach the kidneys. Another dog (Thisbe) received 8 μg./kg. 5-HT into the aorta, showed no change in blood pressure and inhibition of urine flow for one minute ; 40 μg./kg. caused a small rise of blood pressure and another transitory fall in the rate of urine flow (Fig. 5). On both these occasions the reduction in the rate of urine flow seemed to be due to ureteric spasm. In this dog it was unlikely that the 5-HT failed to reach the kidneys, since three consecutive injections of 10 μg. adrenaline given intra-aortically (Fig. 5) immediately before the 5-HT injections caused the type of urinary inhibition always seen after the intravenous injection of adrenaline. In a third dog (Chris) the intra-aortic injection of 36 μg./kg. 5-HT caused a large rise in blood pressure and inhibition of urine flow (Fig. 6).

Administration of 5-HT into the Cubital Vein.—When 5-HT was injected into the malleolar vein the appearance of antidiuresis was correlated to the degree of the respiratory response (see top of p. 37). It was therefore possible that both antidiuresis and panting depended on changes in the vascular bed in the thorax, or on direct stimulation of sensory

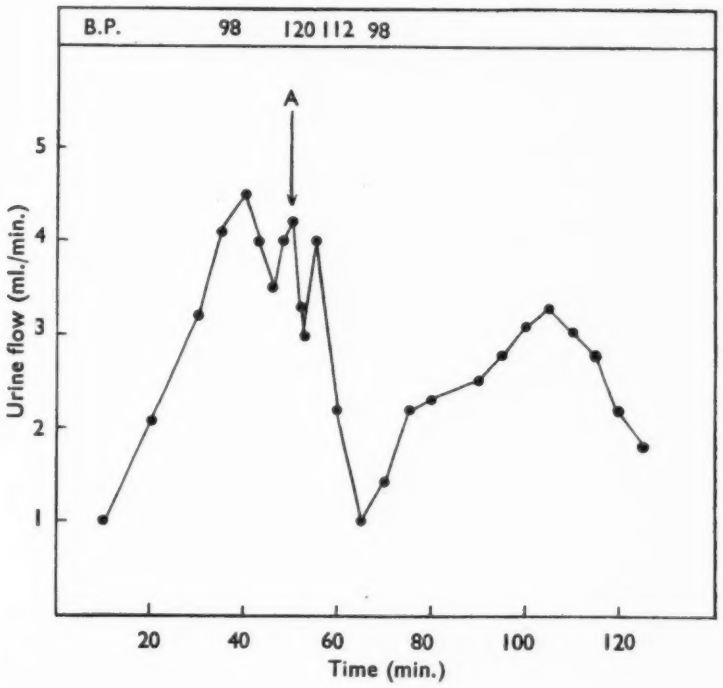

FIG. 6.—Record of water diuresis (Chris). Effect of intra-aortic injection, at A, of 36 μg./kg. 5-HT. At top, blood pressure in mm. Hg.

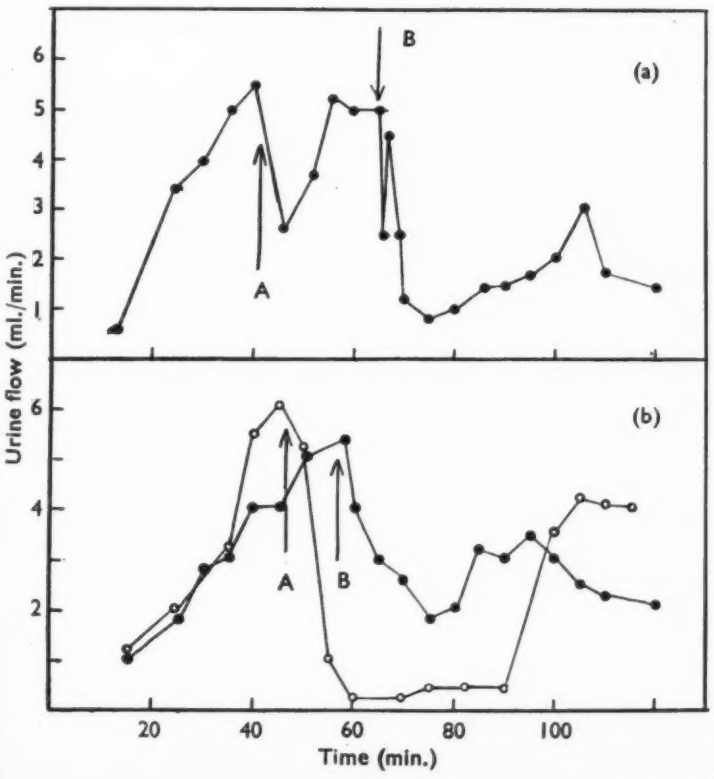

FIG. 7.—Record of water diuresis (Jess). Comparison of the effect of 5-HT injected into the malleolar and cubital veins. (a) At A, 13.4 μg./kg. into malleolar vein; at B, 13.4 μg./kg. into cubital vein. (b) O——O at A, 13.4 μg./kg. into cubital vein; ●——● at B, 13.4 μg./kg. into malleolar vein.

receptors there (Comroe, Van Lingen, Stroud, and Roncorini, 1953; MacCanon and Horvath, 1954; Mott and Paintal, 1953; Schneider and Yonkman, 1953, 1954)—that is, that both phenomena were caused by reflexes arising in the same area. It was also possible that the variability of the response after injection of 5-HT into the malleolar vein depended on the rate at which this substance was picked up by the platelets, and therefore on the final concentration of 5-HT reaching some part of the thoracic viscera. If these hypotheses were valid, then an injection into the cubital vein should be more effective than one into the malleolar, because the path and time to the thorax are shorter; and

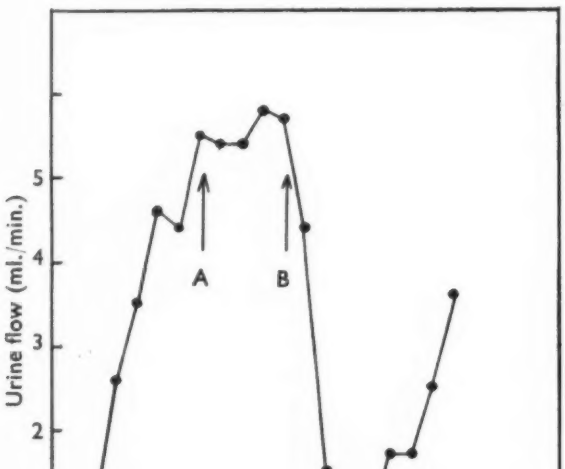

FIG. 8.—Effect on activity of 5-HT of contact with blood before injection into the circulation. Record of water diuresis (Chris). At A, 18 μg./kg. after 16 sec. contact with dog's own blood; at B, 18 μg./kg. without preliminary admixture with blood. Both injections into cubital vein.

5-HT should be inactivated by contact with blood for a short time. It has been confirmed in 5 dogs that a given dose of 5-HT is more effective as an antidiuretic when given into the fore- as compared with the hind-limb vein, and that a smaller dose is effective by the former route (see Fig. 7). Furthermore, a dose injected into the fore-limb vein was more powerfully antidiuretic than twice that amount injected into the aorta earlier the same afternoon (Fig. 5).

Fig. 8 shows the effect of an injection of 5-HT after contact with blood. For the first injection at A, after inserting the hypodermic needle in the cubital vein, 2 ml. blood was withdrawn into the syringe which already contained 18 μg./kg. 5-HT in 1.0 ml. 0.9% NaCl solution. The mixture of blood and 5-HT containing saline was then immediately re-injected. The whole procedure was completed in 16 sec. For the second injection at B, 18 μg./kg. 5-HT in 1.0 ml. 0.9% NaCl solution was given into the same vein without preliminary admixture with blood. In the first instance the change in rate of urine flow was negligible, but in the second there was marked antidiuresis. This shows that contact with blood for 16 sec. is sufficient to reduce the amount of free 5-HT below that necessary to produce antidiuresis. This interval is roughly equal to that between the injection into the hind-limb vein of 5-HT or acetylcholine and the respiratory response—that is, the circulation time from leg to thorax or head in dogs of 12 to 16 kg. body weight is about 14–16 sec.

The Effect of 5-HT on Blood Pressure.—It was evident from the intra-aortic records of blood pressure that in the conscious dog 5-HT produced the same response as in the anaesthetized animal—that is, a sharp rise in pressure lasting 2 to 3 min. and then a gradual fall. In the conscious dog the preinjection level was re-attained in 11 to 13 min. It was also clear that there was no inhibition of urine flow unless there was a change in general blood pressure (Figs. 5 and 6).

The Effect of 5-HT in the Dog with Diabetes Insipidus.—Fig. 9 shows that, in a diabetic dog (Lady), drinking between 5 and 6 l. of water daily, an intravenous injection of 15.5 μg./kg. 5-HT caused a brief inhibition of urine flow, probably due to ureteric spasm, followed by recovery and then a moderate but longer-lasting inhibition. 31 μg./kg. produced a similar response. Unfortunately there is no information about the pre-diabetic response of this dog. Post-mortem histological examination of the hypothalamus and pituitary showed that few supraoptic or paraventricular cells remained. Those cells present, when stained with toluidine blue, were pale and agranular, and when stained with chrome-alum-haematoxylin and phloxin were loaded with dark material. The posterior lobe and median eminence were atrophic.

The Effect of 5-HT after Renal Denervation.—In one dog (Jess) the kidneys were denervated by stripping the renal pedicles and freeing the kidneys from all peritoneal connexions. 11 days later an intravenous injection of 13.5 μg./kg. 5-HT induced antidiuresis.

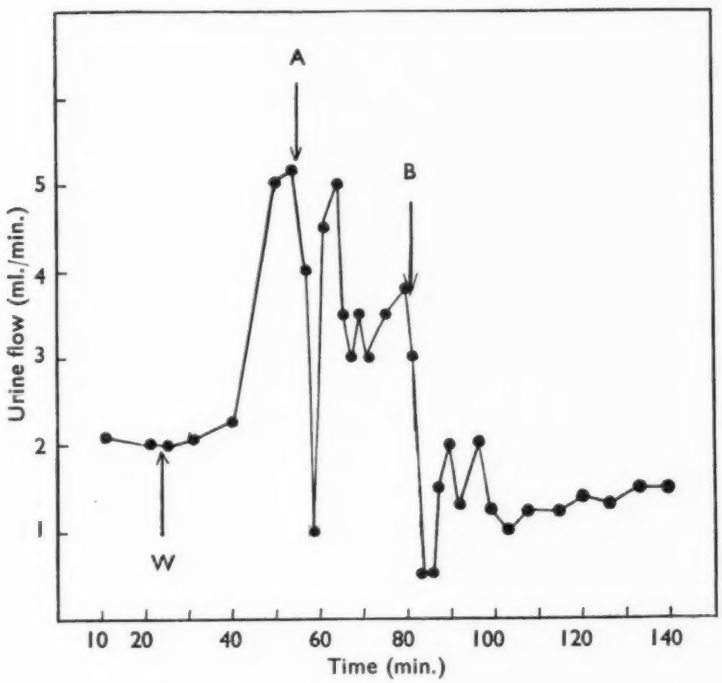

FIG. 9.—Effect of intravenous 5-HT on water diuresis in dog (Lady) with diabetes insipidus. At W, 350 ml. water by mouth. At A, 15.5 μg./kg. 5-HT. At B, 31 μg./kg. 5-HT. Both injections into malleolar vein.

Appearance of the Kidney after the Administration of 5-HT.—Once, immediately before killing an anaesthetized dog (Bouncer), the abdomen was opened and the kidney watched during the injection into the malleolar vein of 26 μg./kg. 5-HT. Some seconds after the injection the surface of the organ paled markedly. The colour began to return 2 to 3 min. later. After waiting 15 min. to allow time for recovery, a further dose of 26 μg./kg. was injected intravenously, followed 1 min. later by a concentrated solution of bromphenol blue in 0.9% NaCl solution. As quickly as possible after this the left renal pedicle was clamped, the kidney removed and bisected, and examined under low-power binocular magnification. Dye was seen plentifully in the medulla, but in only a few radial streaks in the cortex. Juxtamedullary glomeruli were empty of dye. These findings were confirmed later by histological examination of both kidneys.

DISCUSSION

The experiments described show that, in the conscious dog, single intravenous injections of 5-hydroxytryptamine (5-HT) given during water diuresis cause an inhibition of urine flow. In normal dogs, the minimum individual effective dose given into the malleolar vein varied from 10 to 20 μg./kg. In one dog with congenital absence of the right and fibrosis of the left kidney, the minimal effective dose was only 4 μg./kg. In any one dog the degree of antidiuresis induced by a given dose varied from day to day, even under constant conditions, and on some days no antidiuresis would be seen. Subcutaneous doses of up to 74 μg./kg. caused no antidiuresis. These results in the dog are similar to those of Erspamer (1954a and b) working on rats. They differ, however, in the degree of sensitivity of the two species, in the most effective route of administration and in the regularity of response. Erspamer found that in some rats as little as 4 μg./kg. 5-HT given subcutaneously caused antidiuresis. Clearly dogs do not exhibit anything like this degree of sensitivity to subcutaneously administered 5-HT. On the other hand, rats appear to be fairly insensitive to its intravenous administration (Erspamer, 1954b), though we have been unable to trace any published work on the intravenous use of a salt of the pure base in a study on water diuresis in rats.

Measurements of diodone and creatinine clearances in dogs showed that during antidiuresis the values of both RPF and GFR fell rapidly. 20 to 25 min. later RPF rose towards or above pre-injection levels, even though urinary inhibition persisted. The GFR, too, might recover during the phase of urinary inhibition, but usually it remained depressed for a longer period than the RPF. When antidiuresis failed to appear in response to an injection of 5-HT the clearances of

diodone and creatinine rose. Here, then, dogs and rats showed similar reactions, in that, when antidiuresis occurred, it seemed to depend on a reduction in GFR rather than in RPF. That blood pressure rose and vaginal mucous membrane paled when antidiuresis followed injection of 5-HT, and, on the other hand, that clearance values rose when antidiuresis failed to occur, all suggest that vasoconstriction, both generally and in the kidney, is a prerequisite for antidiuresis. In other words, the kidney is no more sensitive to 5-HT than are other areas, and 5-HT, unlike the vasopressor fraction of posterior pituitary extracts, does not specifically induce water reabsorption. This agrees with Erspamer's conclusion that the antidiuresis is vascular in origin, but is contrary to the idea that 5-HT is a specific renal vascular hormone. Sala and Castegnaro (1953) believe it is a water reabsorbing hormone. They used large doses given subcutaneously to dogs, and report that antidiuresis occurred, on occasion, in the absence of any change in GFR. There was, however, always a reduction in RPF. They interpret this as meaning that 5-HT induces specific reabsorption of water. Unfortunately no observations were made on the blood pressure which would be greatly increased by such doses, which would cause complex and variable changes in the kidney and possibly other remote effects such as release of antidiuretic hormone.

Why, in the present experiments, the reaction of the kidney to 5-HT varied from day to day is unknown. There seemed to be no relationship to the size of dose used. The variability may have depended on the proportion of injected 5-HT affecting the circulatory system and the proportion finally reaching the kidney.

No observations were made on the effect of infusions of 5-HT as was done by Corcoran, Masson, Del Greco, and Page (1954). They infused dogs intravenously at rates of 2 to 13 μg./kg./min. 5-HT, and found no hint of antidiuresis with the smaller doses and hypotension with the larger. Judging from the present results the low doses were probably too small to cause antidiuresis, even when given steadily over a period of time, since 5-HT is so rapidly removed from the circulation (Gaddum, Hebb, Silver, and Swan, 1953) that its concentration is unlikely to rise. The higher doses were within the antidiuretic range for some dogs, but hypotension would confuse the issue. The last complication was absent from the experiments described in this paper.

The present results on intracarotid injections in a dog with diabetes insipidus accord with those of Erspamer (1954a, b) and Barac (1953), who concluded that an intact posterior lobe was unnecessary for an antidiuretic reaction to 5-HT administration, and that no direct central stimulation was involved.

Certain observations make it unlikely that 5-HT can be considered a renal hormone. In the dog the uncertainty and variability of its effect on the kidney raise the suspicion that its most important action is at some other site. Compared with the kidney, the response of the systemic blood pressure, the ureter (Abrahams and Pickford, 1956a), and uterus (Abrahams and Pickford, 1956b) show greater consistency and sensitivity. When 5-HT is in contact with blood its activity is rapidly reduced, as was shown by Gaddum, Hebb, Silver, and Swan (1953) and in the present experiments. When 5-HT is given by intravenous injection the time during which it is in contact with blood before it reaches the ureter, uterus or kidney must be nearly the same ; yet the smallest injected dose to which the kidney responds varies from 10 to 20 μg./kg., whereas the other two organs react to doses of 0.5 μg./kg. and 5 μg./kg. respectively—and are thus more sensitive than the most sensitive kidney. Yet it might be expected that a hormone would act on its target organ in lower concentration than that necessary to affect other organs. The systemic blood pressure is altered by doses of 5-HT which are wholly without effect on the rate of urine flow (Abrahams and Pickford, 1956a). Further, when 5-HT is injected into the aorta, and there was reason to suppose that the drug was reaching the kidney, the renal reaction to 40 μg./kg. was insignificant, but there was a marked response to the intravenous injection of 20 μg./kg. (Fig. 5). Furthermore, antidiuresis only occurred when the general blood pressure was raised, and was never seen unless there was evident respiratory reaction to the injection. All these facts made it necessary to look beyond the kidney for the origin of the effect of 5-HT on urine flow. Mott and Paintal (1953) found that 5-HT stimulates vagal fibres whose origins lie between the great veins and left atrium ; and 5-HT is more strongly antidiuretic when injected into the cubital vein instead of the malleolar vein or aorta (Fig. 7). It would thus appear that 5-HT produces antidiuresis mainly by setting up reflexes from the thorax, probably from the pulmonary bed, which, by altering the general blood pressure, affect the kidney.

This does not exclude other modes of action as well. Page (1952) found that the perfused kidney shows vasoconstriction in response to 5-HT, though the reaction is less marked than that to

adrenaline. There is, too, the present observation that the denervated kidney responded by antidiuresis to an injection of 5-HT. This could be explained as due, in part, to the direct vasoconstrictor action of 5-HT ; but also, almost certainly, to the powers of autoregulation displayed by the denervated kidney (Sellwood and Verney, 1955). No substance can strictly be called a renal hormone because it is active on the denervated kidney, unless, at the same time, the general blood pressure is unchanged.

Finally, there is the problem of why the antidiuretic effect of 5-HT sometimes greatly outlasts an effective blood concentration. Verney and Vogt (1938, 1943) found that in some dogs a long-lasting suppression of urine flow followed short periods of renal arterial occlusion. It may be that injected 5-HT, by depriving the renal cortex of blood for as long as 2 or 3 min., which it was observed to do, has much the same effect as renal arterial occlusion. Possibly, too, the sharp changes in blood pressure may cause the release of antidiuretic hormone from the posterior lobe of the pituitary.

Summary

1. Observations were made on conscious dogs of the effect during water diuresis of injecting 5-hydroxytryptamine (5-HT) subcutaneously, intramuscularly, into the malleolar or cubital vein, or the aorta or carotid artery.

2. 5-HT was most effectively antidiuretic when injected into the cubital vein, less so when injected into the malleolar vein or carotid artery, and still less so when given into the aorta at a point above the origin of both renal arteries. It was least effective when administered subcutaneously or intramuscularly.

3. When injected into the malleolar vein the minimum effective antidiuretic dose varied from 10 to 20 μg./kg.

4. Antidiuresis was not regularly seen after a given dose of 5-HT. When it did occur it was accompanied by obvious respiratory reactions. When aortic pressure was measured this was seen to rise at the onset of antidiuresis. The vaginal mucous membrane also paled. If the respiratory and vascular reactions were inconspicuous or absent antidiuresis did not occur.

5. During antidiuresis the renal clearances of diodone and creatinine fell. One or both values might increase towards or above preinjection level before urine flow recovered. When 5-HT failed to cause antidiuresis both clearance values rose.

6. The meaning of the results is discussed ; it is concluded that there are several reasons for the antidiuretic action of 5-HT, but that it is not a specific renal hormone.

We wish to record our gratitude to the Upjohn Company for a generous supply of 5-hydroxytryptamine creatinine sulphate.

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THE EFFECT OF 5-HYDROXYTRYPTAMINE ON THE URETER AND ON THE BLOOD PRESSURE OF DOGS; AND OF ADRENALINE, NORADRENALINE, AND POSTERIOR PITUITARY EXTRACTS ON THE URETER

BY

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When observing the action of 5-hydroxytryptamine (5-HT) on the rate of urine flow in conscious dogs it was often noticed that almost immediately after an intravenous injection of this substance there was a brief marked slowing of urine flow, followed by an equally brief period of rapid flow before the appearance of the long-lasting antidiuresis described by Erspamer (1954) (Fig. 1). These fluctuations in rate of urine flow lasted no more than 2–3 min. and could be most easily explained as due to temporary spasm of the ureters. It was decided, therefore, to examine the response of the ureter *in situ* to 5-HT. Moreover, it was of interest in connexion with the antidiuresis seen after 5-HT to know whether renal function was affected by smaller or greater doses than those which had an action on smooth muscle such as that of ureter, uterus and of the vascular system. At the same time, the opportunity was taken of observing the effect of some other drugs on the ureter, and of the effect of 5-HT on the blood pressure of the anaesthetized dog.

METHODS

Nine dogs were anaesthetized either with chloralose, 0.1 g./kg., or with pentobarbitone sodium, 0.033 g./kg., intravenously. The abdomen was opened through a midline incision and a length of ureter, of 2 inches or more, was prepared for perfusion through its lumen by inserting one cannula into its upper end just below the renal pelvis, and another into its lower end either immediately above the bladder, or, from within the bladder, through the ureteric orifice. Care was taken when tying in the cannulae to leave the main ureteric blood vessels outside the ligatures so that the ureter kept its normal blood supply and, to some extent, its normal innervation. This arrangement made it possible for solutions injected into distant veins to reach the ureter by the normal route. The lumen of the ureter was perfused with 0.9% NaCl solution from a Mariotte bottle connected with one or other cannula, so that the pressure and direction of perfusion could be controlled. Inflow and outflow from the ureter were connected to the exterior by fine rubber tubes

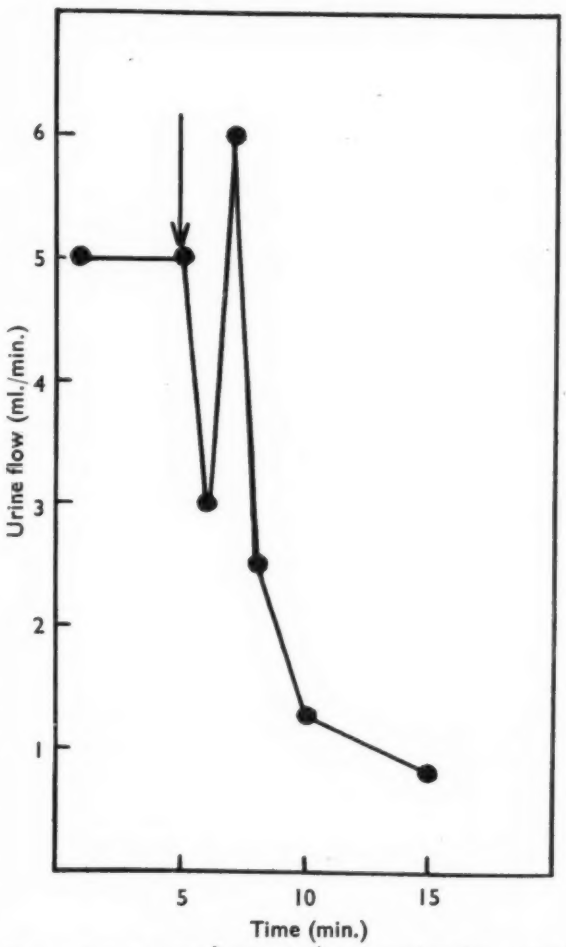

FIG. 1.—Part of a water diuresis curve in a conscious dog. 350 ml. water given by mouth 65 min. before the intravenous injection, at arrow, of 12.7 μg./kg. body weight 5-HT. Ordinate, urine flow in ml./min. Abscissa, time in min.

passing either through the midline incision or through stab wounds in the flank. The rate of flow of fluid through the ureter was measured by means of a drop recorder. Carotid or femoral arterial pressure was recorded from a mercury manometer. The abdomen was closed before observations were begun.

5-HT was administered in the form of the creatinine sulphate, but all doses are calculated and given as the weight of base/kg. body weight. Posterior lobe extracts used were "Pitressin" and "Pitocin" of Parke, Davis & Co. Synthetic (−)-adrenaline and (−)-noradrenaline bitartrate were made up shortly before use in 0.9% NaCl solution in such concentration that the volume injected was never more than 1.0 ml. All injections were made intravenously.

RESULTS

It was found that no fluid passed through the ureter when the perfusion pressure was below a critical level which varied somewhat in different dogs. Usually the limiting pressure was about 20 cm. water, but in some dogs it was as low as 15 cm. Just above the minimum effective pressure, drops appeared at regular intervals in ones, twos or threes. At somewhat higher pressures a number of drops passed through quickly, regularly followed by a short pause (Fig. 2). Further

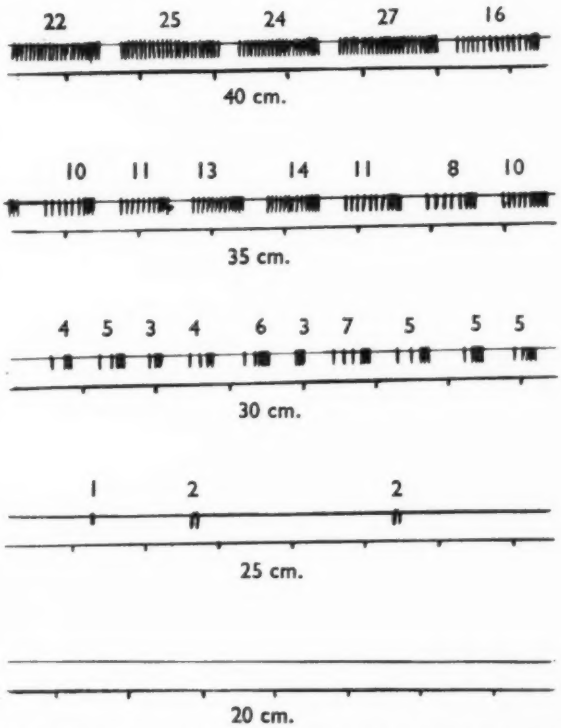

FIG. 2.—Pattern of flow through the ureter at different intralumenal pressures. Upper line is drop record of ureteric flow, the numerals above which indicate the number of drops appearing from the ureter at each outflow-spurt of the perfusion fluid. Lower line, time in 10 sec. Intralumenal pressures in cm. water.

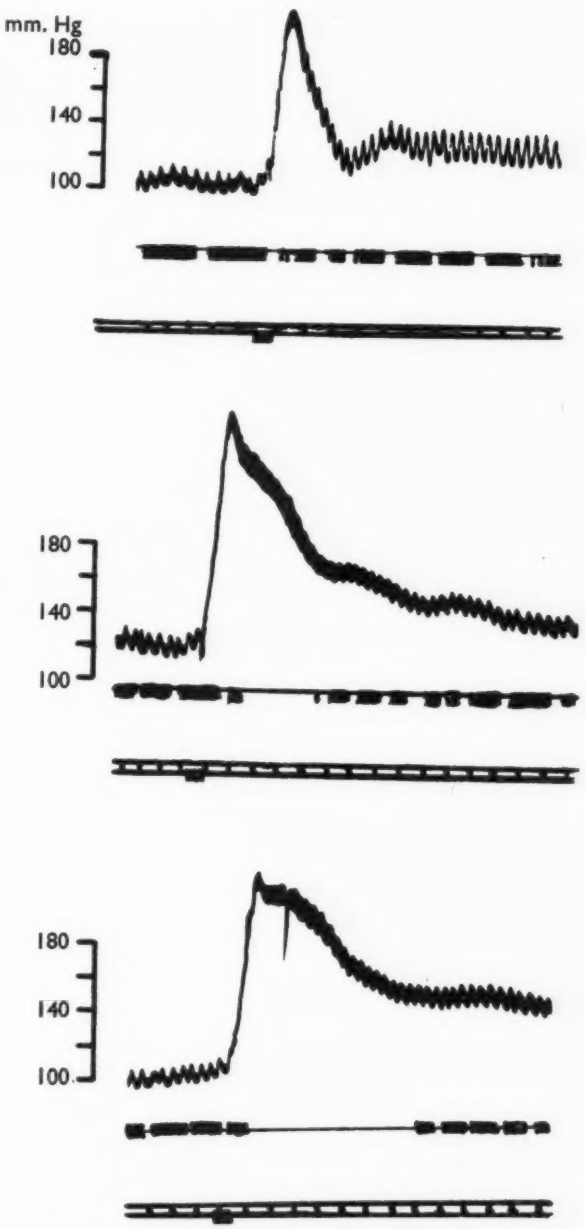

FIG. 3.—Effect of intravenous 5-HT on flow through the ureter, and on arterial pressure. Top record, 3.6 μg./kg. 5-HT; middle record, 10.8 μg./kg. 5-HT; bottom record, 18 μg./kg. 5-HT. In each record: top line, arterial pressure; 2nd line, drop record of ureteric flow; 3rd line, time in 10 sec.; bottom line, signal marker.

increase of perfusion pressure increased the number of drops between pauses until a point was reached when the drops came steadily and continuously. This pressure varied greatly between dogs, ranging from 40–60 cm. water. In any one experiment the pattern of flow through the ureter remained constant for a given perfusion pressure.

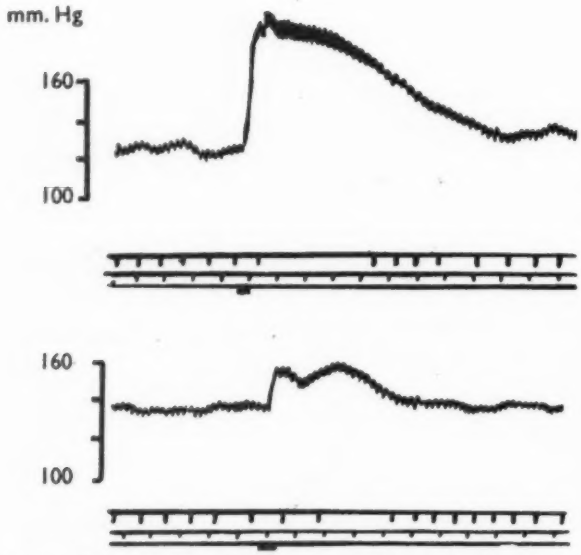

FIG. 4.—Effect of intravenous adrenaline and noradrenaline on flow through the ureter. Upper record, 5 μg. (−)-noradrenaline Lower record, 5 μg. (−)-adrenaline. In each record: top line, arterial blood pressure; 2nd line, ureteric flow; 3rd line, time in 10 sec.; bottom line, signal marker.

Intermittency was not due to resistance in the perfusion system, since at any pressure above zero a steady, more or less rapid, stream of fluid passed. If the blood supply of the ureter was occluded, or if anaesthesia was deep, there was no intermittency of flow, and instead a continuous series of drops emerged from the lower cannula. The results were the same whether chloralose or pentobarbitone sodium was the anaesthetizing agent. On two occasions the ureter was perfused from its lower end. Despite this reversal of direction the pattern of flow was unchanged.

With this information available, observations on the actions of drugs on the ureter were made at perfusion pressures between 25–30 cm. water unless otherwise stated, and only on ureters that showed intermittency of flow.

Effect of 5-HT.—Fig. 3 shows the effect on flow through the same ureter of three different concentrations of 5-HT given into the saphenous vein. With increase in amount of the drug, flow was held up for a longer period. Since, in the conditions of the experiments, the pressure above a ureteric block did not build up as it would have done in natural circumstances (see Fig. 1), no secondary rush of fluid could be expected, and none was seen. The minimum intravenous dose observed to have an effect on the ureter was 3.6 μg./kg. (Fig. 3). It can also be seen from Fig. 3 that the cessation of ureteric flow began just after the onset of the blood-pressure rise, and that flow returned before the blood pressure fell to its initial level. If, during the inhibition of flow caused by 5-HT, the perfusion pressure was quickly raised to 55 or 60 cm. water, then ureteric flow started again immediately.

Effect of Adrenaline and Noradrenaline.—Fig. 4 shows that both adrenaline and noradrenaline caused a short-lasting cessation of transureteral flow which occurred during the period of increased arterial pressure. On one occasion, 2 min. after the intravenous administration of 2 mg. of phentolamine, (2-*N*-*m*-hydroxyphenyl-*p*-toluidinomethyliminazoline, " Rogitine ") neither ureter nor blood pressure reacted to 10 μg. adrenaline.

Effect of Extracts of Posterior Lobe of Pituitary.—Doses of vasopressor extract up to 10 mU. were without effect on the pattern of flow through the ureter. On the other hand, 50 sec. after 50 mU. of the oxytocic fraction had been given there was a temporary decrease in the rate of flow, whilst following 100 mU. the number of drops in a series

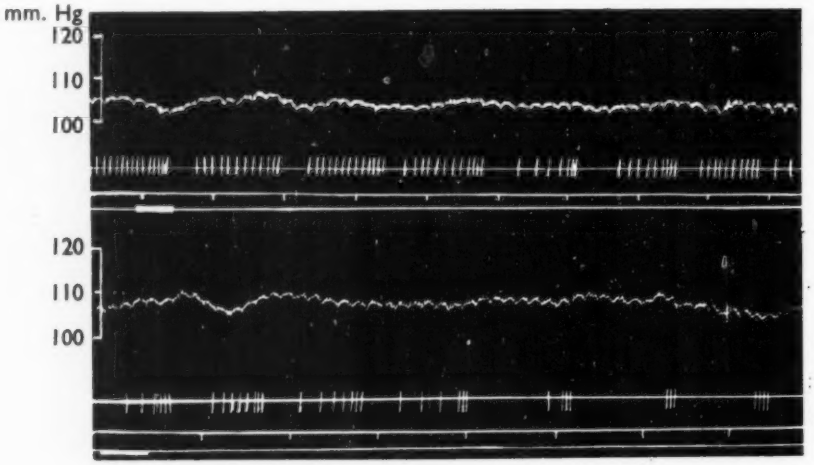

FIG. 5.—Effect of intravenous oxytocin on flow through the ureter. Upper record, 50 mU. at signal; lower record 100 mU. at signal. In each part: top line, arterial pressure; 2nd line, drop record of ureteric flow; 3rd line, time in 10 sec.; bottom line, signal marker.

decreased and the length of the pauses between series increased for more than 5 min. (Fig. 5).

Effect of 5-HT on Arterial Blood Pressure.—In 9 dogs given 41 injections of 5-HT ranging from 3.6 μg. to 84 μg./kg., only twice (Fig. 6) was there any fall of blood pressure. Both occurred in the same dog following the injection of 4 μg./kg. 5-HT. Later, this animal gave a pure

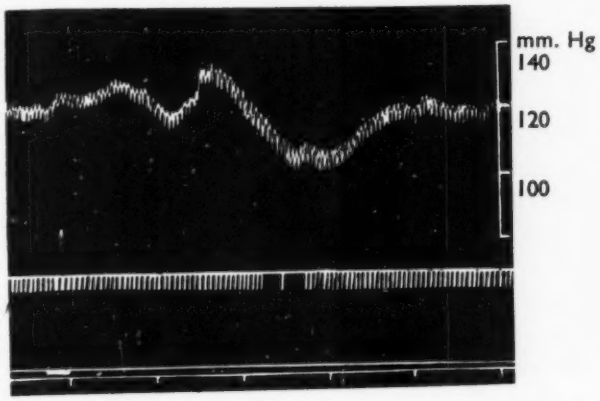

FIG. 6.—Blood-pressure record. Dog, pentobarbitone sodium. At signal, 3.6 μg./kg. 5-HT intravenously. Illustrates the only occasion on which any fall of pressure occurred after the initial rise.

pressor response to the same or larger doses. Two other dogs given 4 μg. and 2 μg./kg. of 5-HT showed only a rise of blood pressure. On all other occasions 5-HT caused an immediate rapid rise in blood pressure which lasted about 60 to 70 sec. The pressure then fell to within 8–10 mm. Hg of the preinjection level, thereafter falling slowly over some minutes. This occurred after moderate as well as after large doses of 5-HT (Fig. 7). Table I gives four examples, picked at random from the records, showing the time before the blood pressure recovered completely. Thus, in anaesthetized dogs, the intravenous administration of 5-HT had a pure pressor action which lasted some minutes. The response to the intracarotid injection of 5-HT was always a marked, but short-lived, pressor effect (Fig. 7).

TABLE I

TO SHOW TIME FOR FALL OF BLOOD PRESSURE TO NORMAL AFTER INTRAVENOUS 5-HT

Four examples picked at random

Dose (μg./kg.)	Time (min.)
7·3	5·0
7·3	3·0
21·0	4·8
49·0	6·0

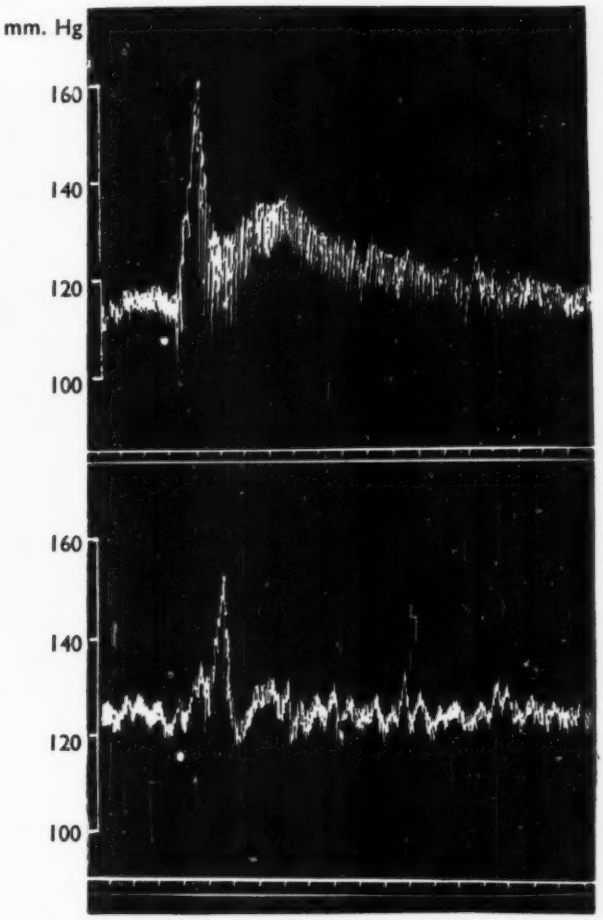

FIG. 7.—Blood pressure record. Dog, pentobarbitone sodium. Comparison of the effect in the same dog of 12.5 μg./kg. 5-HT given into the femoral vein (upper record), and into the carotid artery (lower record).

DISCUSSION

The preliminary observations on ureteric activity showed that between certain perfusion pressures fluid emerged from the lower end of the ureter in small rushes with regular pauses between. Since the abdomen was closed, peristalsis was not actually seen ; but the observations are in accord with clinical and experimental work showing that the ureters have peristaltic activity, and that urine enters the bladder in spurts. Much of the experimental work on the ureter has been done on the excised organ observed in a bath, but Trattner (1932) noted the response of the human ureter *in situ* and found that contractions appeared in it with an intralumenal pressure of 0–12 cm. water and were maximal between 3 and 18 cm. water. He also found that peristalsis disappeared at 38–70 cm. pressure. Lucas (1908) found that a pressure of 15 cm. water applied at the lower end of the isolated

ureter of dogs was not transmitted to a higher level —that is, the muscle was able to remain contracted against this force. Morales, Crowder, Fishman, and Maxwell (1952) observed in dogs with exteriorized trigones that at high rates of urine flow, which presumably means at high renal pelvic pressures, the urine came in a continuous jet and not in spurts. All these pressure values and reactions are similar to those recorded here, namely, that intermittent flow began at about 15 cm. water and became continuous at various pressures from 40 cm. upward. It is surprising that the ureter seems to work as well in reverse as in the normal direction, but Gruber (1930) noticed the same phenomenon in the isolated ureter of pigs. The preparation used may thus be considered a reasonably normal one, and consequently suitable for study of the action of 5-HT and other drugs.

The ureter of the dog was sensitive to as little as 3.6 μg./kg. 5-HT given intravenously, and reacted by a spasm lasting 1 to 2 min. which began almost immediately after injection. It appeared that the ureteric muscle relaxed sooner than that of the vascular system, but it may be that the perfusion pressures used were sufficient to force open the ureter during the phase of recovery from spasm. It seems clear that the rapid swings in rate of urine flow seen immediately after the intravenous injection of 5-HT can be fully explained by its action on the ureter; the contraction holds back the urine, which accumulates in the renal pelvis, so that, when relaxation occurs, a considerable volume is delivered in a short time.

Several workers (Gruber, 1930; Lucas, 1908; Roth, 1917) have found that the isolated ureter responds to adrenaline by an increase in tone or strength of contraction. This accords with the present findings on the intact ureter, that both adrenaline and noradrenaline temporarily inhibit transureteral flow during the phase of greatest increase in arterial pressure.

Ross and Stehle (1930) showed that an intravenous injection of 1 mg. of a laboratory-made posterior pituitary lobe extract had a constrictor action on the ureter that was inadequate to account for antidiuresis. The observations described above show that an antidiuretic dose (10 mU.) of the vasopressor fraction has no perceptible effect on the ureter, whereas the oxytocic fraction, in amounts that may well occur in a limited number of normal circumstances (Abrahams and Pickford, 1954), does cause contraction of the ureteric, as of the uterine, muscle.

With regard to the effect of 5-HT on blood pressure, the results were only in part the same as those obtained by other workers. Only in one dog was there any secondary hypotensive effect. On all other occasions the response was purely pressor, and no late sustained fall of pressure was seen even after large doses (Page and McCubbin, 1953). Why these results were different is unknown. Intracarotid injections of 5-HT caused a sharp rise of blood pressure, with return to normal in about 1 min. The response was immediate, and no secondary fall of pressure was ever seen. Thus 5-HT may have a transient central action (cf. Heymans and Heuvel-Heymans, 1953). That the responses to intracarotid and intravenous injection of 5-HT were somewhat different may depend on the rate at which 5-HT is removed from the circulation (Gaddum, Hebb, Silver, and Swan, 1953).

The object of the observations on blood pressure was not to re-examine the mechanism of action of 5-HT but to determine the dose which, given intravenously, had a measurable effect on the blood pressure, and to compare it with the dose to which other systems or tissues reacted. In Fig. 3 the response of the ureter is clearly a threshold one, yet the pressor response is well developed; thus it may be concluded that the ureter is less sensitive than the arterial pressure to 3.6 μg./kg. The uterus of the conscious and anaesthetized dog contracts in response to an even lower concentration than that which affects the blood pressure (Abrahams and Pickford, 1956a). As described in another paper (Abrahams and Pickford, 1956b), antidiuresis is rarely seen after single intravenous doses of 5-HT of less than 10 μg./kg. Thus, whatever may be the reason or reasons for the hypertensive action of 5-HT, a rise in general blood pressure is provoked by a smaller dose of 5-HT than affects either ureter or kidney, but needs a larger one than causes contraction of the uterus.

Summary

1. In the anaesthetized dog observations were made on the activity of the ureter left *in situ*, with blood supply intact, and the lumen perfused with 0.9% NaCl solution.

2. At perfusion pressures of about 15 cm. water, or more, small volumes of fluid at regular intervals passed through the ureter.

3. At pressures of 40 cm. water and upward, the actual pressure varying with the individual, a continuous series of drops passed through the ureter.

4. Intravenous 5-hydroxytryptamine, by causing contraction of the ureteric muscle, completely prevented the passage of fluid through its lumen for

1 to 2 min. and slowed the flow for a further period of 1 to 2 min.

5. Both adrenaline and noradrenaline caused a transitory contraction of the ureteric muscle.

6. Given in adequate antidiuretic dose, the vasopressor fraction of posterior lobe extract was without effect on the ureter. The oxytocic fraction caused contraction of the muscle.

7. In dogs, anaesthetized with either chloralose or pentobarbitone sodium, 5-hydroxytryptamine rarely caused other than a pure pressor response, whether given into the carotid artery or intravenously.

A few days before our manuscript was submitted a paper appeared in *J. Physiol.* (1955, **129,** 436) on " The Behaviour of the Intact Ureter in Dogs, Rabbits and Rats " by D. W. Gould, A. C. L. Hsieh and L. F. Tinckler. There is no disagreement between the conclusions reached by these authors and by us.

We wish to express our gratitude to the Upjohn Company for a generous supply of 5-hydroxytryptamine creatinine sulphate, and to Messrs. Ciba for a supply of " Rogitine."

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THE EFFECT OF 5-HYDROXYTRYPTAMINE ON THE UTERUS OF CONSCIOUS AND OF ANAESTHETIZED DOGS

BY

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The following observations were made on the response of the uterus of the dog to 5-hydroxytryptamine (5-HT) in order that it might be possible to compare the minimum dose to which this organ reacts with the minimum effective dose for the ureter, the kidney, and the systemic blood pressure, in the same species.

METHODS

Observations were made on conscious and anaesthetized bitches. The former were ovariectomized and provided with a fistula of one uterine horn ; the spontaneous and induced movements of the uterus were recorded by means of a balloon inserted into the lumen. The method has previously been described (Abrahams and Pickford, 1954). Four dogs were observed under anaesthesia which was induced by the intravenous administration of 0.1 g./kg. chloralose in 0.9% NaCl solution. For 7 to 10 days before observation the dogs received 0.25 to 0.5 mg. stilboestrol dipropionate in oil (British Drug Houses preparation) given subcutaneously on alternate days. Two of the 4 animals were subjected to a preliminary operation 2 days before the experiment. One uterine horn was exteriorized so that its movements could be recorded by means of a balloon, as in the conscious dogs. In the other 2 dogs the preliminary operation was omitted, and, instead, uterine contractions were recorded by tying a thread to the upper end of one uterine horn and passing it over pulleys to a lever writing on a kymograph. The lower end of the uterus was fixed by gripping the cervix in volsellum forceps passed up the vagina, the forceps then being clamped to a stand. The abdominal incision was closed round a wide glass tube filled with warm paraffin which guarded the uterine horn and its attached thread. Unless otherwise stated all injections were made into the malleolar vein. 5-HT was administered in the form of the creatinine sulphate dissolved in 0.9% NaCl solution. All doses are calculated and given as μg. base/kg. body weight.

RESULTS

Conscious Dogs.—Observations were made on 3 dogs, whose uteri all showed regular spontaneous contractions. In all 3 the uterus gave the same type of response to an intravenous injection of 5-HT, namely, a contraction which was usually larger than the spontaneous ones, followed by a period of quiescence during which spontaneous contractions were suppressed and tone sometimes diminished (Fig. 1). The minimum effective dose was less than 1 μg./kg.; 17 μg./kg. was a supramaximal dose, since the contraction induced was no larger, nor the inhibition longer in duration, than after the injection of 10 μg./kg. The longest observed period of inhibition was 7 min.

Anaesthetized Dogs.—In all 4 animals, regardless of the recording system used, the response to

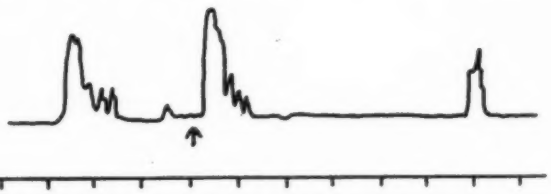

FIG. 1.—Tracing from record of uterine contractions in conscious dog. Effect of 1.4 μg./kg. 5-HT intravenously at arrow. Upward movement indicates contraction. Upper line, uterine contractions. Lower line, time in 50 sec.

the intravenous injection of 5-HT was the same as that of the conscious dog, with the addition that a loss of tone was apparent (Fig. 2). The minimum effective dose in 3 dogs was less than 1 μg./kg., and in one was 0.45 μg./kg. The effect of intravenous and intracarotid injections was compared. An intravenous dose of 3 μg./kg. caused a contraction, followed by an inhibition lasting 5.5 min. The same dose given into the carotid artery caused a contraction followed by an inhibition lasting 2 min.

DISCUSSION

These experiments show that the uterus of the dog, when *in situ*, is highly sensitive to 5-HT and responds to the intravenous injection of 1 μg./kg., or even less, by a contraction, followed by some minutes' inhibition. No tachyphylaxis was shown

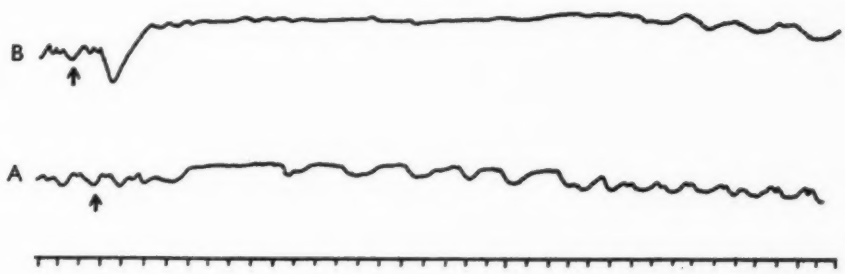

FIG. 2.—Tracing from record of uterine contractions in anaesthetized dog. Effect of intravenous administration of 5-HT. Downward movement indicates contraction. Upper two lines, uterine contractions. Lowest line, time in 10 sec. In A, 0.45 μg./kg. 5-HT at arrow; in B, 2.5 μg./kg. 5-HT at arrow.

to injections given repeatedly at 10 to 15 min. intervals. The action of 5-HT appears to be wholly peripheral, since intravenous injections were more effective than intracarotid ones, and the results were the same whether or not the dog was anaesthetized. The response of the uterus differs from that of the ureter, since, in the latter, there was no sign of any inhibitory phase following the contraction of the muscle (Abrahams and Pickford, 1956a). It is clear that the uterus of the dog is more sensitive to intravenous injections of 5-HT than is the ureter, kidney or general blood pressure (Abrahams and Pickford, 1956a and b).

SUMMARY

1. The effect of 5-HT on the uterus of dogs was observed, both in conscious animals and in those anaesthetized with chloralose.

2. In response to an intravenous injection of 5-HT the uterus gave a contraction which was often greater than those occurring spontaneously. This was followed by a period of inhibition which might last for as long as 7 min.

3. In all dogs tested the minimum effective intravenous dose was less than 1 μg./kg. In one dog it was 0.45 μg./kg.

We wish to record our gratitude to the Upjohn Company for a gift of 5-HT.

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Brit. J. Pharmacol. (1956), **11**, 52.

INACTIVATION OF ADRENALINE AND NORADRENALINE BY HUMAN AND OTHER MAMMALIAN LIVER *IN VITRO*

BY

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In a previous paper, Bain, Gaunt, and Suffolk (1937) described a biological assay method for following the course of adrenaline inactivation *in vitro*. Using this method they showed, among other things: (1) that adrenaline was only partially inactivated in cats' defibrinated blood, and that this was largely owing to the slow passage of some of the adrenaline into the red cells, whence most of it could be recovered by laking; (2) that the addition of tissue slices, particularly of liver, to a blood-adrenaline mixture, determined a relatively rapid and complete inactivation of the adrenaline.

In some subsequent work, not published in full, Bain and Dickinson (1938) showed that the mean rate of adrenaline inactivation by liver slices, as indicated by the half-inactivation time, varied with the species: it was fastest for the guinea-pig and for man, slowest for the cat, dog, and mouse, and intermediate for the rat. The results from 16 samples of human liver were reported at that time. Three of these inactivated adrenaline so slowly as to seem in a different category from the others. As two of the subjects had suffered from arterial hypertension of non-renal origin it was suggested that delayed inactivation of the transmitter of adrenergic vasomotor nerve activity—then thought to be adrenaline—might be responsible for such forms of raised arterial blood pressure.

The importance of noradrenaline was not, of course, recognized when these observations were made. It seemed desirable, therefore, in the light of subsequent events, to compare the behaviour of noradrenaline with that of adrenaline. This has accordingly been done by repeating some of the earlier work, but in parallel experiments in which both drugs were investigated at the same time under the same conditions. Most experiments were with human liver.

A preliminary note on some of the results has already appeared (Bain and Batty, 1952).

MATERIALS AND METHODS

Drug Solutions.—A concentrated stock solution of (−)-adrenaline hydrochloride was made by dissolving a known amount of the synthetic *laevo* base (British Drug Houses, Ltd.) in the calculated amount of 0.1 N-HCl, and adjusting the final volume with distilled water so that the solution contained 10 mg. base/ml. From this a dilute stock solution, containing 1 mg. base/ml., was prepared as required. A standard adrenaline solution, containing 10 μg. base/ml., was freshly prepared for each assay from the dilute stock solution.

*Laevo*noradrenaline was at first used as the hydrochloride, and later as the bitartrate monohydrate—both kindly supplied by Dr. M. L. Tainter. A stock solution, containing 1 mg. base/ml., was prepared by dissolving the appropriate amount of the salt in distilled water (1 mg. base = 1.22 mg. hydrochloride = 1.99 mg. bitartrate). Standard solutions for the assays were prepared by dilution of this stock solution.

Stock solutions were kept in the refrigerator. During the course of an assay the standard solutions were kept cool on ice.

Blood-Liver-Amine System.—Apart from a few preliminary experiments, where the inactivation of the amines in blood alone was studied, the inactivating system consisted of defibrinated blood, liver slices, and amine.

The advantages of blood as the medium in such experiments have been pointed out before (Bain *et al.*, 1937); the chief of these is the very consistent behaviour of adrenaline in it—a consistency far surpassing that in any other medium tried which could be regarded as physiological in any real sense. Furthermore, it seems obvious that the integrity of the liver cells, and hence the behaviour of the tissue slices, are more likely to approximate to the normal conditions in blood than in any artificial medium. Cat blood was always used, since the assays were done on cats.

Guinea-pig, rat, and mouse livers were taken from newly killed animals. Several mouse livers were

needed to provide sufficient material for an experiment. Cat liver was got from the animals used to supply blood for the experiments.

Specimens of human liver were obtained from the post-mortem room within 24 hr. of death and were kept in the refrigerator if there was to be any delay in using them. Up to 3 days' storage in the refrigerator does not seem to alter appreciably the amine inactivating power of liver. On one occasion an inactivation curve was repeated on a sample of human liver after 2 days', and on another after 3 days', refrigeration. In neither did the inactivation rates before and after storage differ. This agrees with some earlier results of Bain (1937, unpublished), who, in addition, found no difference between biopsy and post-mortem specimens, and that the activity of a sample of human liver removed at biopsy was not altered after 24 hr. in the refrigerator. These points suggest that observed differences in the inactivating power of different samples of human liver are real, and are not due simply to post-mortem changes.

The liver was cut into thin slices with a razor. The slices were dried between filter paper and weighed out into 1.0 g. portions. To each of the required number of hard-glass vaccine bottles, suspended in a water bath at 37° C. and containing 4.8 ml. defibrinated blood, was added a 1.0 g. portion of liver slices. At the appropriate time—which became zero time for that particular tube—0.2 ml. of the dilute solution of adrenaline or noradrenaline was added, giving, in the fluid phase of the system, an initial concentration of 40 μg. base/ml. (This concentration, though unphysiological, is convenient for assay purposes, since only very small volumes of blood are needed for the early parts of the assay, when the sensitivity of the test animal is at its lowest; and only moderate volumes are needed later when, although most of the amine may have been destroyed, the sensitivity of the test animal is likely to be higher, so that small amounts can be estimated (Bain *et al.*, 1937).) The amount of amine remaining was determined at intervals thereafter. The vaccine bottles were agitated mechanically throughout the experiment, except when samples were being drawn off for assay.

Method of Assay.—This was an extension, to include noradrenaline, of the method of "continuous assay" described in detail for adrenaline by Bain *et al.* (1937).

Several—usually three—different standard doses of each amine were used. These were injected in rotation, at regular intervals of about 3 min., into the jugular vein of a spinal cat, and the pressor responses recorded from the carotid artery. When the amount of adrenaline or noradrenaline in an experimental solution had to be determined, the injection of one of the standard solutions was replaced by the experimental one.

Since the concentration of adrenaline or noradrenaline in the experimental solutions is continually changing, bracketing of test and standard responses is impossible, and matching occurs only occasionally and by chance. In principle, then, the method consists in the determination of dose-response-time curves, with the frequent interpolation—or rather substitution—of test solutions. The adrenaline or noradrenaline content of any particular test injection is found by reference to the appropriate dose-response-time curve. In practice, and in the most favourable conditions, once an assay is established and is running smoothly alternate injections of standard and test solutions can be given, so that it is possible to estimate an "unknown" about every 6 min.

Part of a typical assay is illustrated in Fig. 1. The left-hand record is from an early stage of the experiment, before the assay proper has started: that on the right is from a much later stage, when the assay was well under way, the sensitivity of the animal had increased, and frequent injections of unknowns were being given.

For further details about technique the 1937 paper by Bain and his colleagues should be consulted.

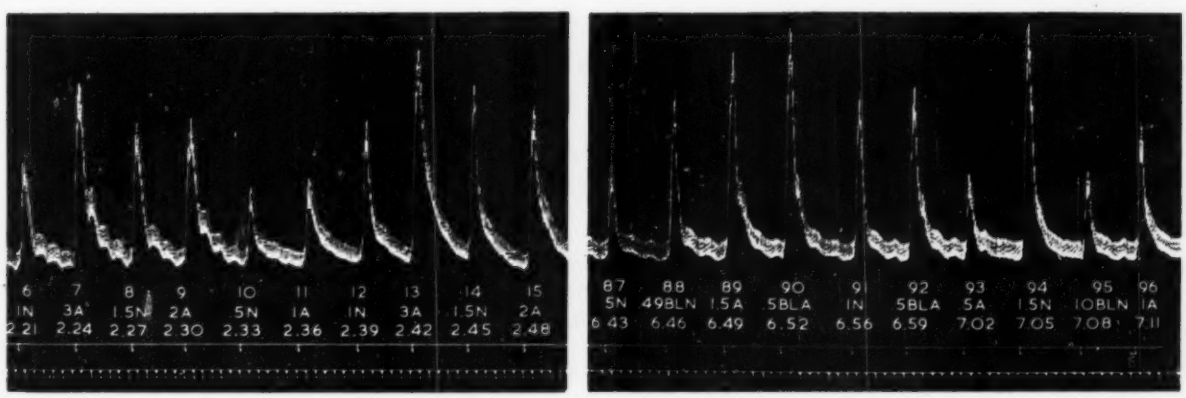

FIG. 1.—Portions of record from typical assay—on left from early stage, and on right from late stage of expt. Below each blood-pressure rise are, in order: no. of injection; material injected—μg. adrenaline (A) or noradrenaline (N), or ml. blood from blood-liver-adrenaline (BLA) or blood-liver-noradrenaline (BLN) mixture; time of administration. The signal marks indicate moment of injection, and time in $\frac{1}{2}$ min., respectively. Note how sensitivity of test animal rises in early part of expt. and has reached a steady level in later record.

RESULTS

Inactivation of Adrenaline and Noradrenaline in Blood.—Before going on to study inactivation by liver, it seemed desirable to do some preliminary experiments to see if noradrenaline added to blood alone behaved in the same way as did adrenaline. This was done in six sets of paired experiments, in each of which the inactivation of adrenaline and of noradrenaline was followed for several hours in defibrinated blood at 37° C. The initial concentration in all experiments was 40 μg./ml.

Both amines behaved similarly, the activity of the blood-noradrenaline mixtures diminishing slowly till they reached, after 2–3 hr., steady values ("equilibrium value") beyond which no further inactivation took place. The average equilibrium value for noradrenaline was 14.0 μg./ml. and for adrenaline 16.3 μg./ml.

This equilibrium phenomenon, apparently first noticed by Sugawara (1928–9), has already been investigated in some detail by Bain *et al.* (1937) for adrenaline. The interest of the present experiments is simply in the demonstration that noradrenaline behaves in the same way.

Bain and his colleagues also showed, however, that the adrenaline activity given by the equilibrium value did not represent all the adrenaline in the blood: a further amount was held inactive in association with the red cells, whence it could be released by laking. To determine if this also happened with noradrenaline, equilibrium mixtures were laked with distilled water and the amine activity again determined.

In three such experiments the average equilibrium concentration of noradrenaline was 14.7 μg./ml., or 36.7% of the initial concentration. A further 22.4% of the original amount was released on laking, giving a total noradrenaline activity representing 60% of the original amount. This is less than the 80% recovery of adrenaline reported in the earlier paper. It was matched, however, by a 57% adrenaline recovery here (probably the laking was inefficient) and so can be taken to indicate that in this further respect—the taking up of the amine by the red cells—noradrenaline behaves like adrenaline.

Inactivation of Adrenaline and Noradrenaline by Liver Slices in Blood.—When adrenaline or noradrenaline is added to blood containing liver slices inactivation of the amine proceeds in an exponential fashion, so that there is a linear relationship between the logarithm of the concentration and time. By constructing such a linear graph by eye, from the assay data for each experiment, the time for destruction of half the adrenaline or noradrenaline is readily determined. The result of one such experiment (No. 65) is illustrated in Fig. 2.

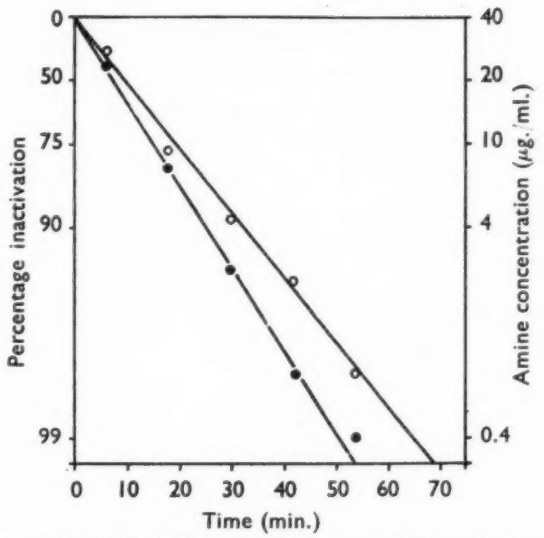

FIG. 2.—Results of single expt. (No. 65) comparing inactivation of adrenaline and noradrenaline by human liver. The regressions were drawn by eye through the experimental points. Ordinates, inactivation (%), and amine concentration (μg./ml.). Abscissa, time in min. Open circles, adrenaline; closed circles, noradrenaline. The speed of inactivation of both amines was exceptionally fast in this expt.

The average half-inactivation times, obtained in this way for different mammalian species, are summarized in Table I. It is clear that noradrenaline is destroyed more rapidly than adrenaline by the liver of each species.

TABLE I

AVERAGE HALF-INACTIVATION TIMES (MIN.) OF ADRENALINE AND NORADRENALINE WHEN INCUBATED WITH LIVER OF VARIOUS SPECIES IN BLOOD AT 37° C.

Numerals in parentheses indicate the lowest and highest values obtained.

Species	No. of Expts.	Adrenaline	Noradrenaline
Guinea-pig ..	7	15·1 (11·0–22·0)	11·2 (9·0–15·0)
Rat	9	25·3 (18·5–46·0)	19·2 (12·0–28·0)
Cat	7	45·1 (25·0–72·0)	27·6 (12·0–50·0)
Mouse	2	50·5 (50·0–51·0)	36·5 (30·0–43·0)
Man	28	16·5 (9·5–27·0)	13·1 (7·5–24·0)

Furthermore, the results with adrenaline are in good agreement with those previously published (Bain and Dickinson, 1938). The half-inactivation

time of 13 min. for human liver given in that paper was, however, derived from the 13 liver samples presumed to be "normal." When the values for the slow livers are included the average becomes 15.5 min., with a range of from 10 to 27 min. The present results are in striking accord with these.

Course of Adrenaline and Noradrenaline Inactivation.—The relatively large number of experiments with human liver made it possible to analyse these results in more detail. Before this was done, however, the precise relation between log concentration and time in a blood-liver-amine system was determined.

For this purpose the standard type of experiment was carried out in triplicate for both the amines, with human liver as the inactivator. The replicate experiments with adrenaline were designated A_1, A_2, and A_3, and those with noradrenaline N_1, N_2, and N_3. The amine was added to the appropriate tube at a planned time, and samples were taken for assay from each tube 6, 18, 30, 42, and 54 min. later. The estimated amine contents at these times in the six experiments are shown in Table II.

TABLE II

ADRENALINE AND NORADRENALINE CONCENTRATIONS (μG./ML.) ESTIMATED BIOLOGICALLY IN REPLICATE INACTIVATION SYSTEMS AT DIFFERENT TIMES (MIN.) AFTER ADDITION OF THE AMINES

Expt.	Time from Addition of Amine (min.)				
	6	18	30	42	54
A_1	30·0	8·9	4·1	1·8	0·8
A_2	28·6	8·0	—	2·6	0·6
A_3	28·5	10·8	4·7	2·2	1·0
N_1	25·7	9·0	3·1	1·1	0·4
N_2	21·0	6·4	—	0·4	—
N_3	24·0	7·3	2·9	0·9	0·3

In analysing the results the methods described by Finney (1950) were used. The data for adrenaline and noradrenaline were treated independently to determine the relation of log concentration and time, but were combined in order to compare the inactivation of the two amines. The concentrations in Table II were converted to their natural logarithms. The missing values were estimated and the degrees of freedom adjusted accordingly.

The analyses of variance for the replicate experiments with adrenaline and with noradrenaline are shown in Tables III and IV respectively. It is clear from these analyses that there is a linear relation between log concentration and time for each amine.

Furthermore, a t test applied to determine the difference between the two regressions gave $P<0.001$. The difference in slopes of the regressions of log concentration on time is thus highly significant: under identical conditions, therefore, the inactivation of each amine progresses at a different rate.

Regression Coefficients as a Measure of Inactivation Rates.—Having established the linear relation

TABLE III

ANALYSIS OF VARIANCE OF THE DATA FROM REPLICATE EXPERIMENTS ON ADRENALINE INACTIVATION BY HUMAN LIVER

Source of Variation	Sum of Squares	d.f.	Mean Square	Variance Ratio	P
Regressions ..	22·514	1	22·514	833·85	<0·001
Deviations from regression ..	0·191	3	0·064	2·37	0·1–0·2
Between times ..	22·705	4	5·676	210·22	<0·001
,, expts.	0·076	2	0·038	1·41	>0·2
Residue ..	0·186	7	0·027		
Total ..	22·967	13			

TABLE IV

ANALYSIS OF VARIANCE OF THE DATA FROM REPLICATE EXPERIMENTS ON NORADRENALINE INACTIVATION BY HUMAN LIVER

Source of Variation	Sum of Squares	d.f.	Mean Square	Variance Ratio	P
Regressions ..	36·611	1	36·611	892·95	<0·001
Deviations from regression ..	0·072	3	0·024	0·59	>0·2
Between times	36·683	4	9·171	223·68	<0·001
,, expts.	0·708	2	0·354	8·63	0·02–0·03
Residue ..	0·245	6	0·041		
Total ..	37·636	12			

TABLE V

THE REGRESSION COEFFICIENTS FROM THE LOG CONCENTRATION-TIME RELATION FOR ADRENALINE AND NORADRENALINE INCUBATED WITH HUMAN LIVER

Expt. No.	b Adrenaline	b Noradrenaline
21	−0·02378	−0·02582
22	−0·04467	−0·07530
24	−0·03882	−0·05131
25	−0·03949	−0·05060
27	−0·02382	−0·03045
28	−0·04075	−0·03590
31	−0·03576	−0·04594
32	−0·06222	−0·07941
33	−0·04192	−0·04873
34	−0·04323	−0·05254
36	−0·06400	−0·07163
37 (a)	−0·04368	−0·07289
37 (b)	−0·05128	−0·07481
40	−0·07163	−0·05860
41	−0·05342	−0·05223
43	−0·06611	−0·06656
44	−0·04740	−0·06271
46	−0·03915	−0·07023
49	−0·03325	−0·04611
51	−0·06145	−0·07234
55	−0·03330	−0·05346
57	−0·02516	−0·04163
58	−0·02737	−0·03873
59	−0·02882	−0·04094
61	−0·05560	−0·06228
63 (a)	−0·05478	−0·06110
63 (b)	−0·03858	−0·05237
65	−0·07244	−0·08886
Mean ..	−0·04474	−0·05655

TABLE VI
ANALYSIS OF VARIANCE OF REGRESSION COEFFICIENTS FROM 28 EXPERIMENTS WITH HUMAN LIVER

Source of Variation	Sum of Squares	d.f.	Mean Square	Variance Ratio	P
Between drugs	0·00196	1	0·00196	39·2	<0·001
,, expts.	0·01073	27	0·00039	7·8	<0·001
Residue ..	0·00136	27	0·00005		
Total ..	0·01405	55			

between log concentration and time for both amines, it was possible to analyse the data from the experiments with human liver using the regression coefficient as a measure of the inactivation rate. Table V gives the regression coefficients from the 28 experiments mentioned earlier. Analysis of these (Table VI) shows a highly significant difference between the rates of inactivation of the two amines, noradrenaline being the more rapidly destroyed. (It is perhaps worth emphasizing, however, that it is the constancy of the difference, rather than its magnitude, that contributes so greatly to its significance.)

The mean regressions are shown graphically in Fig. 3. The calculated half-inactivation times, and their fiducial limits ($P=0.95$), are 15.5 (13.8–17.7) min. for adrenaline and 12.25 (11.1–13.7) min. for noradrenaline. The corresponding values derived by inspection of the graphs drawn by eye for each experiment are 16.5 min. for adrenaline and 13.1 min. for noradrenaline (Table I).

Mechanism of Adrenaline and Noradrenaline Inactivation by Human Liver.—An enzyme system was thought to be responsible for the amine inactivating property of liver, because boiling for a few minutes destroyed this property. Of the three enzyme systems known to inactivate adrenaline and noradrenaline, only amine oxidase (Blaschko, Richter, and Schlossmann, 1937) and cytochrome oxidase (Keilin and Hartree, 1936, 1937, 1938) were considered, for the third—catechol oxidase—is probably not important in mammals (Bhagvat and Richter, 1938). As amine oxidase is inhibited by octyl alcohol and is unaffected by cyanide, whereas cytochrome oxidase is inhibited by cyanide and is unaffected by octyl alcohol, the presence of either enzyme—or of both—can readily be determined.

Control systems, consisting of blood, liver, and amine in the usual quantities, were compared with similar systems which contained in addition either 10^{-3}M-sodium cyanide or octyl alcohol.

The results with cyanide are shown in Table VII. The addition of cyanide had no marked effect upon the rate of inactivation of either amine. Octyl alcohol, on the other hand, caused a profound slowing of inactivation: in one experiment with adrenaline the destruction at 309 min. in the inhibited system was comparable to that at 33 min.

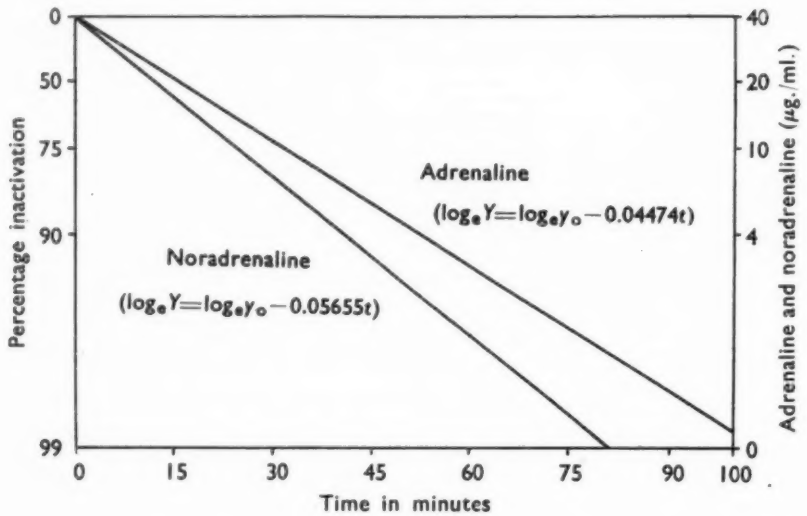

FIG. 3.—Regressions of log concn. on time for adrenaline and noradrenaline inactivation by human liver, calculated from the data of Table V. Upper line, adrenaline; lower line, noradrenaline.

TABLE VII
EFFECT OF CYANIDE ON THE HALF-INACTIVATION TIMES OF ADRENALINE AND NORADRENALINE BY THREE SAMPLES OF HUMAN LIVER

Expt.	Adrenaline		Noradrenaline	
	Without Cyanide	With Cyanide	Without Cyanide	With Cyanide
1	12·25 min.	12·5 min.	10·75 min.	8·5 min.
2	14·0 ,,	14·5 ,,	11·5 ,,	12·0 ,,
3	16·5 ,,	17·0 ,,	12·75 ,,	13·0 ,,

in the control; in the other it was equivalent at 154 min. in the presence of octyl alcohol to that at 15 min. in the control. The results with noradrenaline were similar.

It thus seems clear that the enzyme responsible for the inactivation of adrenaline and noradrenaline in these experiments was amine oxidase.

Discussion

It was hoped, in embarking on these experiments, that it would be possible either to corroborate or disprove the hypothesis (Bain and Dickinson, 1938) that delayed inactivation of the transmitter of adrenergic nerve activity might be responsible for some forms of raised arterial tension. The clinical data available about the patients were, however, quite inadequate for this purpose. Furthermore, there was no evidence that the results with human liver were not normally distributed: the earlier results of Bain and Dickinson might therefore have occurred by chance. Finally, though there is evidently a fairly wide variation in the rate of amine inactivation by different samples of liver, it is not known whether there is a corresponding variation at or near the endings of adrenergic nerves. Nor is it easy to see how the state of affairs at these endings could be determined.

In any event it is obvious that the original hypothesis, based on results with adrenaline, can now, whatever its worth, be extended to include noradrenaline.

Summary

1. When noradrenaline is added to defibrinated blood, its rate of disappearance, and its distribution between cells and serum, are similar to that of adrenaline.

2. Noradrenaline is inactivated slightly more rapidly than adrenaline when incubated in blood with liver slices of guinea-pig, rat, cat, mouse, and man.

3. There is a linear relation between log concentration and time for the inactivation of adrenaline and noradrenaline by human liver.

4. Analysis of the regression coefficients for adrenaline and noradrenaline inactivation by human liver confirms the more rapid inactivation of noradrenaline.

5. The enzyme responsible for the inactivation is amine oxidase.

Our thanks are due to the Medical Research Council for an expenses grant to one of us (W. A. B.), and to Dr. J. G. Dare for helpful discussions on some statistical problems.

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CHOLINE PHENYL ETHERS AS INHIBITORS OF AMINE OXIDASE

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The parent compound of this series, choline phenyl ether,

was first studied by Hunt and Renshaw (1929), who showed it to be a powerful ganglion stimulant. Hey (1952), while investigating the nicotine-like stimulant activities of nuclear-substituted choline phenyl ethers, noticed that choline *p*-tolyl ether (TM6), which had a very weak ganglion-stimulant action, potentiated the effects of adrenergic nerve stimulation, as indicated by an increase in the tone and amplitude of contraction of the nictitating membrane in response to preganglionic stimulation of the cervical sympathetic nerve (Fig. 1). The compound was also found to potentiate the effects of injected adrenaline and noradrenaline on the blood pressure (Fig. 2) and on the nictitating membrane of cats.

It seemed unlikely that these potentiations were related to actions at ganglia ; they could, however, be accounted for—at least in part—if the compound delayed the destruction of adrenaline and noradrenaline. Work on adrenaline inactivation was then in progress in the laboratory, and we were soon able to show that choline *p*-tolyl ether was in fact a powerful inhibitor of adrenaline inactivation *in vitro*, and that this was because it inhibited amine oxidase. Other related compounds which produced similar potentiating effects were also shown to inhibit amine oxidase.

Whether amine-oxidase inhibition fully accounts for the phenomena which prompted us to look for it will not be considered further here. But, having been led to the discovery of these new inhibitors, it seemed worth while to make a quantitative study of their relative potencies, and to see if we could determine any relations between chemical structure and amine-oxidase-inhibitory activity. This paper describes that work.

By a method about to be detailed, we have sought to correlate amine-oxidase-inhibitory activity and chemical structure in the parent compound and in eighteen nuclear-substituted choline phenyl ethers, and in some related compounds in which the cationic head was modified, and the chain length and structure were altered. We have also compared the activities of these new drugs with well-known inhibitors of amine oxidase such as ephedrine, amphetamine, and cocaine. The nature of the inhibition—whether competitive or non-competitive—has also been determined.

Amine oxidase has usually been studied *in vitro* by manometric methods—for example, by Hare (1928) ; Blaschko, Richter, and Schlossmann (1937b) ; Philpot (1940) ; and Tickner (1951). Blaschko, Richter, and Schlossmann (1937a), however, followed biologically the inactivation of adrenaline by liver slices and liver extracts, assaying the remaining adrenaline on the cat blood pressure and the rabbit intestine. The method used here to determine the degree of inhibition of amine oxidase depends upon the biological assay of adrenaline, and is a modification of that used by Bain, Gaunt, and Suffolk (1937) to follow the inactivation of adrenaline by mammalian tissue slices *in vitro*.

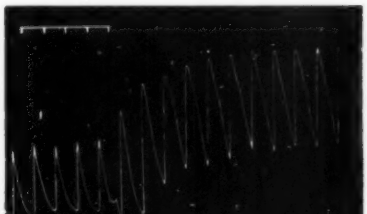

FIG. 1.—Showing the increase in tone, and amplitude of contraction, of the electrically stimulated nictitating membrane produced by the intravenous injection of 10 mg. choline *p*-tolyl ether bromide (TM6), in a cat under chloralose anaesthesia. Preganglionic stimulation 5 sec. in each minute. Time, min.

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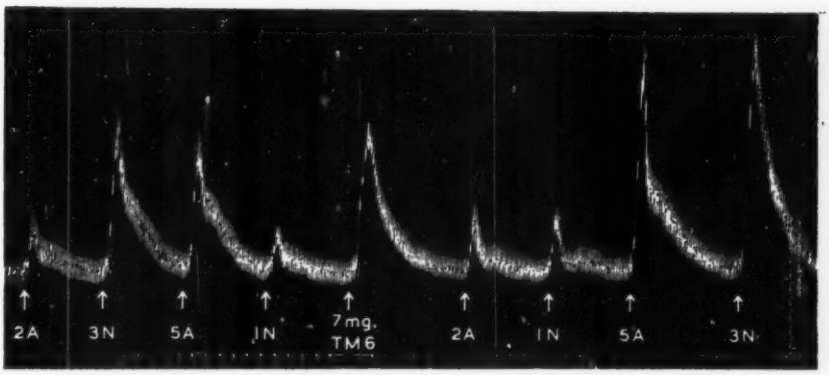


FIG. 2.—Showing the increase in response of the carotid blood pressure to adrenaline and noradrenaline after the intravenous injection of 7 mg. choline *p*-tolyl ether bromide (TM6). A, μg. adrenaline; N, μg. noradrenaline. Spinal cat. Time 30 sec. The immediate pressor effect of TM6 results from ganglionic stimulation (Hey, 1952).

Through the kindness of Dr. Blaschko it has been possible to compare some of the results obtained by this biological assay technique with those obtained by the more conventional manometric method.

A short account of some of the observations has already been published (Brown and Hey, 1952). Since then Schayer and his colleagues (Schayer, Wu, Smiley, and Kobayashi, 1954) have shown, in studies with ^{14}C-labelled adrenaline and other substrates, that choline *p*-tolyl ether is a potent inhibitor of amine oxidase in intact mammals.

METHODS

Adrenaline was incubated with liver slices in defibrinated cat blood at 37.5° C. The adrenaline concentration was determined at various times by assaying the mixture on the blood pressure of spinal cats. Bain *et al.* (1937) used, as a measure of the rate of adrenaline inactivation, the time taken to inactivate half of the original amount present; Bain and Batty (1952, 1956) took the regression coefficient of log adrenaline concentration against time. We have estimated the rate of inactivation by calculating the amount of adrenaline destroyed in 30 min. To do this, four or more blood and adrenaline samples are removed for assay at times ranging from 5 to about 90 min. The adrenaline assay and the estimation of the adrenaline content of the mixtures are described in detail by Bain *et al.* (1937) and by Bain and Batty (1956). When the log adrenaline concentration is plotted against time, as in Fig. 3, a straight line results. The regression coefficient of log adrenaline concentration against time is then calculated; from this the amount of adrenaline remaining after 30 min., and thus the amount destroyed in that time, is determined.

The activity of a compound as an inhibitor of amine oxidase is found by comparing the amount of adrenaline destroyed in 30 min., in the presence of the inhibitor, with the amount destroyed during the same time in its absence. This difference is calculated as a percentage reduction, and it is this which is taken as a measure of amine-oxidase-inhibitory activity. The collection of blood and the preparation of liver slices are described in the paper by Bain *et al.* (1937).

Adrenaline Solutions.—A concentrated stock solution containing 10 mg./ml. of adrenaline base was prepared by dissolving 100 mg. of the base in 5.46 ml. of 0.1 N-HCl and adjusting the volume to 10 ml. with distilled water. From this a dilute stock solution was made containing 1 mg./ml. of adrenaline base.

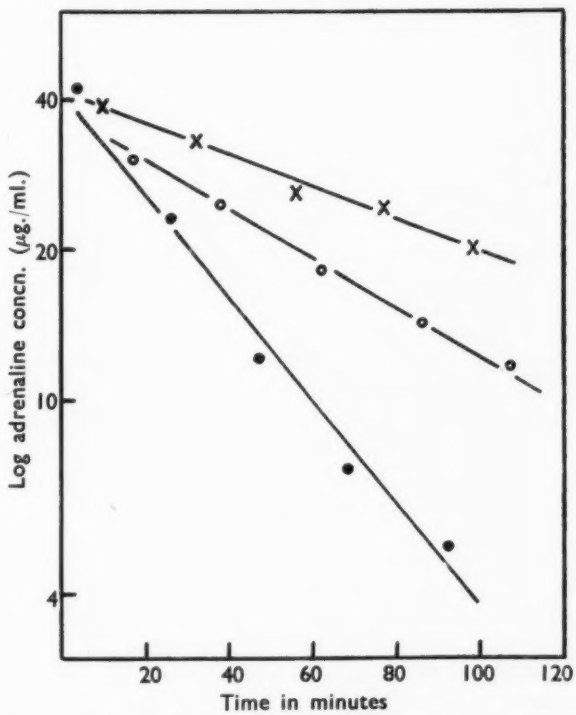


FIG. 3.—The destruction of adrenaline by guinea-pig liver slices and its inhibition by two nuclear-substituted choline phenyl ethers. Initial adrenaline concentration 40 μg./ml. Ordinate, remaining adrenaline concentration, assayed on the blood pressure of the spinal cat; abscissa, time in min. after the addition of adrenaline. Control liver, ●——●; liver plus choline 2: 4-xylyl ether (TM9) 10 μg./ml., O——O; liver plus choline 4-chlorophenyl ether (TM2) 10 μg./ml., X——X.

Inhibitor Solutions.—To determine the inhibitory activity of the choline phenyl ether compounds at concentrations of 10 and 100 μg./ml., solutions were prepared containing 200 μg./ml. or 2 mg./ml. respectively. For the weaker inhibitors, such as ephedrine and cocaine, solutions containing up to 20 mg./ml. were necessary.

Blood-Liver-Adrenaline Mixtures.—The composition of these mixtures in all experiments, unless stated otherwise, was : 4.55 ml. defibrinated cat blood ; 1.0 g. sliced guinea-pig liver ; 0.2 ml. adrenaline solution containing 1 mg./ml. base. In addition, each control liver system contained 0.25 ml. 0.9% NaCl solution, and each experimental system 0.25 ml. of the appropriate inhibitor solution. These mixtures were placed in 25 ml. Jena glass vaccine bottles and incubated in a water-bath at 37.5° C. with constant mechanical agitation.

Other Methods.—Some other methods, used in experiments to determine particular points, are described at the most appropriate places in the text.

Compounds Studied.—These are listed in Tables I, III, and IV.

Results and Discussion

The Effects of Structural Changes in the Choline Phenyl Ether Molecule

The following changes were made in the parent compound :

(1) substitution in the benzene nucleus

(2) modification of the cationic head

(3) alteration of the side chain between the ether oxygen and the nitrogen atom.

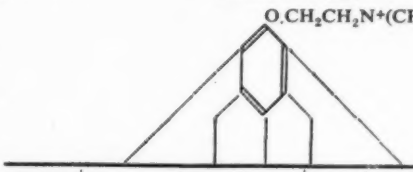
Substitution in the Benzene Nucleus.—The structures of the eighteen nuclear substituted compounds studied, together with their activities, are shown in Table I. Seventeen have various combinations of methyl groups and the halogens chlorine and bromine ; the remaining one has an *iso*propyl group in the *ortho* position. For convenience in experimental work, code names and numbers have been given to the compounds ; these are indicated in Table I, and the compounds are hereafter referred to by these codes.

With the exception of the *iso*propyl derivative, there is a highly significant difference between compounds, taking 0.05% limits of probability ($P<0.001$). For most of the compounds, however, there were insufficient observations to make possible a direct comparison of one with another ; they were therefore treated as groups, so that those having only *ortho* substituents were compared with those having only *meta* or *para* substituents. The difference in activity between those compounds having one *ortho* or one *para* group is insignificant ($P=0.8$–0.9), as is also the difference between

Table I

THE ACTIVITIES OF NUCLEAR-SUBSTITUTED CHOLINE PHENYL ETHERS AS INHIBITORS OF ADRENALINE INACTIVATION BY GUINEA-PIG LIVER

Initial concentration of adrenaline 40 μg./ml. Concentration of inhibitors 10 μg./ml.



Code Number (TM)	2	3	4	5	6	% Reduction in Amount of Adrenaline Destroyed in 30 Min.
5		Br		Br		6·0
20	$CH(CH_3)_2$			CH_3		7·5
3		Cl				12·5
8		CH_3		CH_3		14·0
4		Br				17·5
10	CH_3				CH_3	19·5
7		CH_3				19·5
13		CH_3	CH_2			24·5
14	CH_3	CH_3				25·0
9	CH_3		CH_2			26·0
1						28·0
19	CH_3			CH_3		33·0
16	Cl		Cl			36·5
6			CH_3			37·5
15			Br			38·0
12	Cl					38·5
18	CH_3					40·0
17	CH_3		CH_2		CH_3	40·5
2			Cl			64·5

those with one or with two *meta* groups ($P=0.2$). When, however, compounds having an *ortho* or *para* group are compared with those having one or two *meta* groups, there is a highly significant difference between the two ($P<0.001$). The activity of those compounds possessing an *ortho* or *para* group is significantly different from those with a *meta* group and an *ortho* or *para* group ($P<0.001$).

These results are summarized in Table II, from which it will clearly be seen that *ortho* or *para* substitution increases the activity of the compounds over that of the parent compound, choline phenyl ether (TM1) ; that *meta* substitution depresses it ; and that when *ortho*, or *para*, and *meta*

Table II

THE EFFECTS OF NUCLEAR SUBSTITUTION ON THE AMINE-OXIDASE-INHIBITORY ACTIVITY OF CHOLINE PHENYL ETHER

Activity is expressed as the percentage decrease in the amount of adrenaline destroyed in 30 min. by guinea-pig liver slices in presence of 10 μg./ml. of inhibitor

Substitution	Amine-oxidase-inhibitory Activity
Ortho or *para*	39·5
None	28·0
Ortho or *para*, and *meta*	27·5
Meta	18·5

groups are combined in the same molecule the activity becomes that of the unsubstituted compound.

Comparison of the activity of the compound having an *ortho-iso*propyl group (TM20) with that of the corresponding methyl substituted compound (TM19) shows that the introduction of a larger group into the *ortho* position has markedly reduced the activity.

Modification of the Cationic Head.—The cationic head was changed in two ways ; the modified compounds, together with their code names and numbers and amine-oxidase-inhibitory activities, are shown in Table III.

(i) Changing the trimethyl cationic head to triethyl, as in TE2 and TE6, leads to an approximate tenfold decrease in activity compared with the corresponding trimethyl compounds TM2 and TM6 of Table I.

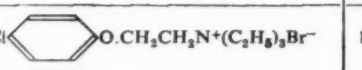
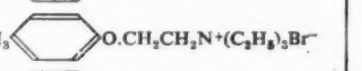
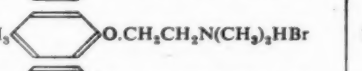
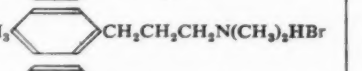
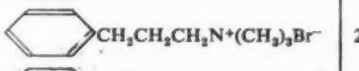
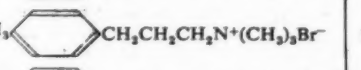
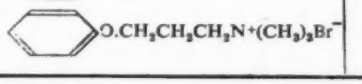
(ii) The effect of replacing the quaternary ammonium cationic head with a tertiary amino group, as in TTM6, is also to reduce the activity to one-tenth that of the corresponding compound in the original series.

Alteration of the Side Chain.—These compounds are also shown in Table III.

TABLE III

THE EFFECT OF ALTERATIONS IN THE CATIONIC HEAD AND CHAIN STRUCTURE OF CHOLINE PHENYL ETHER ON AMINE-OXIDASE-INHIBITORY ACTIVITY

Activity is expressed as the percentage reduction in the amount of adrenaline destroyed in 30 min. by guinea-pig liver slices

Code Name and No.	Formula	Amine-oxidase-inhibitory Activity at Concentrations (μg./ml.) of	
		10	100
TE2		16·5	34·5
TE6		4·5	33·0
TTM6		19·0	35·5
TDM6		5·0	63·0
DM1		20·0	42·0
DM6		12·0	42·0
CTM1		1·0	37·5

(i) Replacement of the ether oxygen by a methylene group—DM1 and DM6—reduces the inhibitory activity on amine oxidase to approximately one-tenth that of TM1 and TM6.

(ii) An increase in chain length of one methylene group, as in CTM1, also reduces the activity considerably, though not so drastically as do changes in the cationic head of the molecule.

The other tertiary amino compound, TDM6, which has a modified side chain, can be compared with the corresponding quaternary compound DM6. There is little difference in the activities of these two compounds—suggesting that the reduction in their activity, as compared with TM6, results from the modification in the side chain rather than from the change in the cationic head.

These results may be summarized by saying that all the structural modifications mentioned here have resulted in compounds with lower activities against amine oxidase than had the original choline phenyl ether series. On the other hand, none of the modified compounds is completely devoid of activity.

It is possible to draw from the observations certain general conclusions concerning the relations between structure and amine-oxidase-inhibiting power in derivatives of choline phenyl ether. Thus, a cationic head consisting of three methyl groups is apparently necessary for maximum activity. Replacing the three methyl groups with three ethyl groups, or changing the quaternary ammonium group to a tertiary amino group, reduces the activity to approximately one-tenth of that of the compound having a trimethyl cationic head. A side chain of the structure $-O.CH_2CH_2-$ also confers maximum activity, though replacement of the ether oxygen by a methylene group does not drastically reduce the activity, nor does increasing the straight chain length by one methylene group. Finally, in the choline phenyl ether series itself, maximum activity is conferred by halogen or methyl group substitution in the *ortho* or *para* position. *Meta* substitution, and the introduction of larger groups in the *ortho* position, appears to reduce rather than to increase the activity relative to the unsubstituted compound.

Comparison with Established Inhibitors of Amine Oxidase

The following compounds have been shown by previous workers to inhibit amine oxidase *in vitro*: ephedrine (Blaschko *et al.*, 1937b); amphetamine (Blaschko, 1940); cocaine and procaine (Philpot, 1940). We have studied these compounds by our method in concentrations ranging from 10 μg./ml.

to 1 mg./ml. These results are shown in Table IV, in which is also included an active inhibitor of the choline phenyl ether series, TM6.

TABLE IV

THE MEAN ACTIVITIES OF SOME ESTABLISHED INHIBITORS OF AMINE OXIDASE, TOGETHER WITH THAT OF CHOLINE *P*-TOLYL ETHER BROMIDE

Activity is expressed as the percentage reduction in the amount of adrenaline destroyed by guinea-pig liver slices in 30 min.

Inhibitor	Amine-oxidase-inhibitory Activity at Concentrations (μg./ml.) of					
	3	10	30	100	300	1,000
Choline *p*-tolyl ether Br (TM6) ..	31·5	37·5	54·5			
Amphetamine H_2SO_4		15·5	33·5	61·0		
Ephedrine HCl ..			4·0	12·5	36·5	
Procaine HCl				9·5	19·5	46·0
Cocaine HCl				1·0	11·5	31·0

It will be seen that 10 μg./ml. TM6 causes an inhibition of 37.5%, and 30 μg./ml. amphetamine sulphate an inhibition of 33.5% ; hence, weight for weight, TM6 is approximately three times more active. Similarly, 300 μg./ml. of ephedrine hydrochloride is required to produce an inhibition equivalent to 10 μg./ml. of TM6, which is therefore thirty times more active. Procaine and cocaine are even less active than ephedrine, 1 mg./ml. of cocaine hydrochloride being needed to give an inhibition equal to that produced by 3 μg./ml. of TM6. Thus, choline *p*-tolyl ether bromide has approximately three times the amine-oxidase-inhibitory power of amphetamine sulphate, thirty times that of ephedrine hydrochloride, and three hundred times that of procaine hydrochloride and of cocaine hydrochloride.

The Nature of the Inhibition

The method of Lineweaver and Burk (1934) was used to determine whether the inhibition of amine oxidase by the choline phenyl ethers is competitive or non-competitive. The velocity of the reaction in which adrenaline is destroyed was investigated at various concentrations of substrate in the presence of different concentrations of inhibitor.

The substrate concentrations in a typical experiment were 40, 100, 200, and 300 μg./ml. of adrenaline. To obtain these concentrations slight modifications were necessary in the composition of the blood-liver-adrenaline mixtures previously described. As before, 4.55 ml. of cat blood was used ; the amounts of adrenaline solution added were, respectively, 0.2 ml. of solution containing 1 mg./ml. base ; 0.05 ml. of 10 mg./ml. solution ; 0.1 ml. of 10 mg./ml. solution ; and 0.15 ml. of 10 mg./ml. solution. The control liver system was made up to a volume of 5 ml. with 0.9% NaCl solution. To the experimental systems were added 0.25 ml. of the appropriate inhibitor solution and sufficient 0.9% NaCl to bring the final volume to 5.0 ml.

The inhibitor used was choline 2:4:6-mesityl ether bromide (TM17) ; the velocity of the reaction was taken as the amount of adrenaline destroyed in 30 min.

Fig. 4 illustrates such an experiment, in which the concentration of inhibitor was 10 μg./ml. From this and other similar experiments, there is no doubt that the substrate-concentration reaction-velocity curves for the destruction of adrenaline by guinea-pig liver, in the absence and in the presence of inhibitor, are not parallel, but are convergent and meet at the ordinate. It is thus clear that the inhibition is competitive.

Manometric Experiments

The activities of TM6 and amphetamine sulphate as inhibitors of amine oxidase were compared by a manometric technique, and the nature of the inhibition by TM6 was determined.

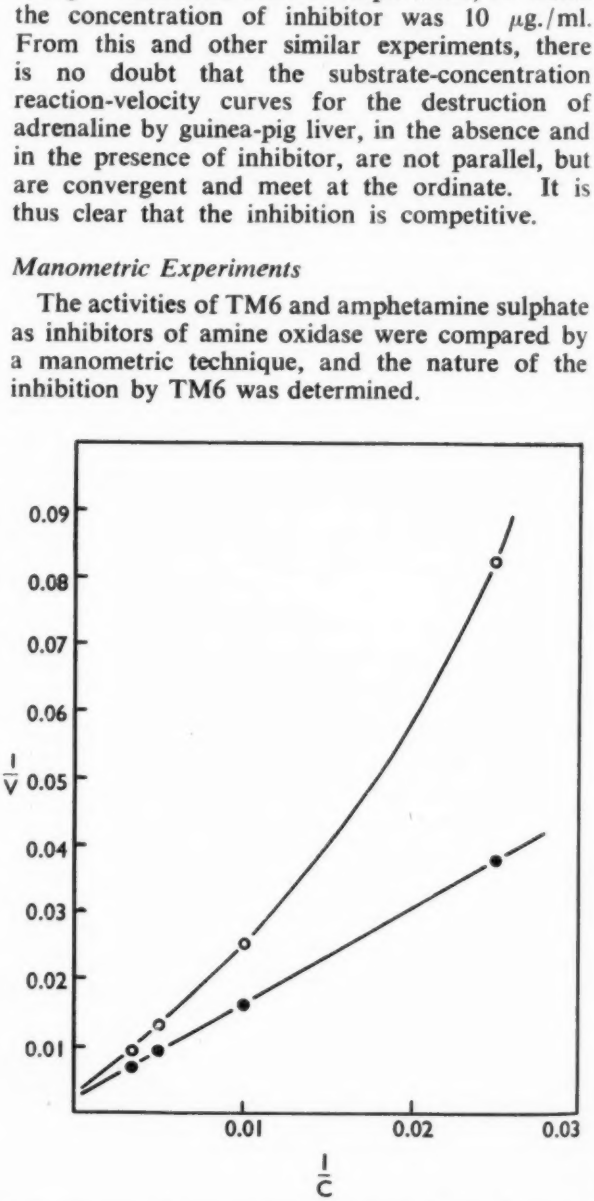


FIG. 4.—Showing the competitive nature of the inhibition of amine oxidase by choline 2: 4: 6-mesityl ether bromide (TM17). The method used is that described by Lineweaver and Burk (1934), the reaction velocity being taken as the amount of adrenaline destroyed by guinea-pig liver slices in 30 min. Ordinate, reciprocal of the reaction velocity; abscissa, reciprocal of the substrate concentration. Control liver, ●——●; liver plus TM17 10 μg./ml., O——O.

In the experiments in which the two inhibitors were compared, a homogenate of guinea-pig liver was used, since this was the tissue employed in the work described earlier in this paper. The nature of the inhibition by TM6 was determined with a homogenate of rabbit liver. The preparation of the extract was the same in both instances. The tissue was frozen solid and then ground to a thin paste, to which was added twice its weight of 0.066M-sodium phosphate buffer *p*H 7.4. This mixture was dialysed overnight against running water; the next morning one-tenth its volume of 0.2M-sodium phosphate buffer, *p*H 7.4, was added. Open manometers and Warburg manometer flasks were used, the centre tubes of which contained 0.3 ml. N-KOH. The gas phase was oxygen and the temperature 37.5° C. The substrate was 0.01M-(−)-*p*-sympatol hydrochloride. The rate of oxidation was followed manometrically, readings being taken at 5 min. intervals for 30 min. The inhibition was measured as the percentage reduction in oxygen uptake at the end of 30 min. In each experiment six manometer flasks were used (1–6); the main chamber of each contained 0.7 ml. of liver homogenate and 0.9 ml. of 0.066M-sodium phosphate buffer *p*H 7.4; the volume was made up to 1.8 ml. with either 0.2 ml. of distilled water, in flasks 1 and 2, or with 0.2 ml. of inhibitor solution in flasks 3, 4, 5, and 6. The side bulbs of flasks 1, 3, and 5 contained 0.2 ml. of distilled water; those of 2, 4, and 6 contained 0.2 ml. of 0.1M-(−)-*p*-sympatol HCl.

A Comparison of Amphetamine and TM6.—Table V records the inhibition produced by various concentrations of TM6 and amphetamine. The 55% inhibition produced by 3.2×10^{-4}M-amphetamine lies midway between those of 77% and 28% produced by 10^{-4}M- and 10^{-5}M-TM6

TABLE V

TO SHOW THE PERCENTAGE REDUCTION OF OXYGEN UPTAKE AFTER 30 MIN. IN GUINEA-PIG LIVER EXTRACT-INHIBITOR MIXTURES

Substrate 0·01M-(−)-*p*-sympatol

Inhibitor	Concentration (M)			
	$3{\cdot}2 \times 10^{-4}$	10^{-4}	$3{\cdot}2 \times 10^{-5}$	10^{-5}
Amphetamine H_2SO_4	55		9	
Choline *p*-tolyl ether Br (TM6)		77		28

respectively. Therefore, the concentration of TM6 having the same effect as 3.2×10^{-4}M, or 59 μg./ml., amphetamine is approximately 5×10^{-5}M, or 13.7 μg./ml. Thus amphetamine has, weight for weight, only one-quarter the activity of the choline ether. By the biological method described earlier, the choline ether inhibitor was found to be three times more active than amphetamine. There is thus close agreement between the results obtained by the two methods.

The Nature of the Inhibition.—An extract of rabbit liver was incubated with two concentrations of substrate, 0.02M- and 0.002M-(−)-*p*-sympatol HCl, alone and in the presence of the inhibitor, 10^{-3}M-TM6. The oxygen consumption was measured over a period of 22 min., at which time the percentage reduction in oxygen uptake in the presence of inhibitor was calculated.

TABLE VI

THE NATURE OF THE INHIBITION OF RABBIT LIVER AMINE OXIDASE BY CHOLINE *P*-TOLYL ETHER BR. (TM6)

Substrate	Extra O_2 Consumption (μl.) after 22 min.		% Inhibition
	Substrate Alone	Substrate + 10^{-3}M-TM6	
0·02M-(−)-*p*-sympatol HCl	29	22	24
0·002M-(−)-*p*-sympatol HCl	13	4	69

Table VI shows that the degree of inhibition is dependent on the concentrations of both inhibitor and substrate. This confirms the earlier observations made by the biological method, that inhibition by choline phenyl ethers is competitive.

CHEMICAL SECTION

Melting and boiling points are uncorrected.

1. 2-aryloxyethyl bromides were prepared by reacting the appropriate phenol in alkaline solution with ethylene dibromide and these were then condensed with trimethylamine in acetone solution, or with triethylamine, to yield the quaternary compound as described in a previous paper (Hey, 1952). New 2-aryloxyethyl bromides are recorded in Table VII, and new quaternary compounds in Table VIII.

2. The following compounds were also prepared:

(*a*) *3(p-tolyl)propyltrimethyl Ammonium Bromide.*—2(*p*-tolyl)propionic acid was prepared by methods described in the literature and converted to the methyl ester by diazomethane. The ester was quantitatively reduced to 3(*p*-tolyl)propyl alcohol by lithium aluminium hydride; b.p. 136°/16 mm. $[n]_D^{20°}$ 1.5329. (Found: C, 80.0; H, 9.70. $C_{10}H_{14}O$ requires C, 80.0; H, 9.40%.) (cf. Brown, 1951.) The above alcohol was treated with an ice-cold solution of phosphorus tribromide in carbon tetrachloride and gave a good yield of the bromide as a colourless liquid b.p. 127°/18 mm. $[n]_D^{20°}$ 1.5412. (Found: C, 56.6; H, 6.26;

TABLE VII

CHARACTERISTICS OF ARYLOXYBROMIDES

Compound R–O–CH_2CH_2Br R =	Characters		Analysis (%)		
			C	H	Halogen
2: 4 Xylyl	Solid; m.p. 45° B.P. 152°/16 mm.	Found Calc. for $C_{10}H_{13}OBr$	52·8 52·4	5·84 5·72	33·4 34·9
3: 4 „	Solid; m.p. 44° B.P. 149°/16 mm.	Found Calc. for $C_{10}H_{13}OBr$	52·7 52·4	5·75 5·72	33·6 34·9
2: 3 „	Liquid; b.p. 148°/15 mm. $[n]_D^{20}$ 1·5478	Found Calc. for $C_{10}H_{13}OBr$	53·0 52·4	5·81 5·72	32·6 34·9
2: 5 „	Liquid; b.p. 135°/14 mm. $[n]_D^{20}$ 1·5421	Found Calc. for $C_{10}H_{13}OBr$	53·2 52·4	5·76 5·72	32·5 34·9
2-Chlorophenyl ..	Liquid; b.p. 153°/15 mm. $[n]_D^{20}$ 1·5700	Found Calc. for C_8H_8OBrCl	41·0 40·8	3·55 3·42	47·6 49·0
4-Bromophenyl ..	Solid; m.p. 61° B.P. 114°/0·8 mm.	Found Calc. for $C_8H_8OBr_2$	34·3 34·3	2·98 2·90	54·1 57·1

TABLE VIII

CHARACTERISTICS OF SUBSTITUTED CHOLINE PHENYL ETHERS AND RELATED COMPOUNDS

Compound (and Code No.) R^1–O–$CH_2CH_2N(R^2)_3Br$ R^1 =	R^2 =	M.p.	Analysis (%)				
				C	H	N	Halogen
4-Chlorophenyl (TE2) ..	Ethyl	142°	Found Calc. for $C_{14}H_{23}ONBrCl$	50·3 49·9	6·84 6·89	3·97 4·16	34·1 34·3
4-Tolyl (TE6)	„	141°	Found Calc. for $C_{15}H_{26}ONBr$	52·4 53·3	6·80 6·63	4·05 4·03	23·4 22·9
2: 4 Xylyl (TM9) ..	Methyl	191°	Found Calc. for $C_{13}H_{22}ONBr$	52·6 54·2	7·30 7·69	4·75 4·86	27·9 27·7
2-Chlorophenyl (TM12)	„	143°	Found Calc. for $C_{11}H_{17}ONBrCl$	44 9 45·0	5·63 5·84	4·56 4·77	38·9 39·3
3: 4 Xylyl (TM13) ..	„	191°	Found Calc. for $C_{13}H_{22}ONBr$	54·0 54·2	7·50 7·69	4·75 4·86	27·9 27·7
2: 3 Xylyl (TM14) ..	„	222°	Found Calc. for $C_{13}H_{22}ONBr$	54·0 54·2	7·57 7·69	4·75 4·86	27·9 27·7
4-Bromophenyl (TM15)	„	196°	Found Calc. for $C_{11}H_{17}ONBr_2$	38·8 39·0	5·05 5·05	3·98 4·13	45·1 47·1
2: 4 Dichlorophenyl (TM16)	„	176°*	Found Calc. for $C_{11}H_{16}ONBrCl_2$	40·4 40·1	5·01 4·90	4·22 4·26	45·3 45·8
2: 5 Xylyl (TM19) ..	„	187°	Found Calc. for $C_{13}H_{22}ONBr$	54·3 54·2	7·79 7·69	4·74 4·86	27·9 27·7

* Jones, Metcalfe, and Sexton (1949) quote m.p. 88°–89°.

Br, 37.1. $C_{10}H_{13}Br$ requires C, 56.3; H, 6.09; Br, 37.5%.) This bromide treated with an excess of trimethylamine in acetone solution at room temperature yielded the required quaternary compound m.p. 116°. (Found : C, 57.1; H, 7.90; N, 5.17; Br, 29.6. $C_{13}H_{22}NBr$ requires C, 57.4; H, 8.13; N, 5.16; Br, 29.3%.)

(*b*) *3(p-tolyl)propyldimethylamine Hydrobromide.*—3(*p*-tolyl)propyl bromide (above) condensed at room temperature with excess dimethylamine in ethereal solution yielding the above tertiary amine as a colourless liquid b.p. 114°/15 mm. $[n]_D^{20°}$ 1.4994. (Found : C, 82.2; H, 10.9; N, 8.07. $C_{12}H_{19}N$ requires C, 81.3; H, 11.0; N, 7.93%.) The hydrobromide had m.p. 149° C.

(*c*) *2(p-toloxy)ethyldimethylamine Hydrobromide.*—2(*p*-toloxy)ethyl bromide condensed at room temperature with excess dimethylamine in ethereal solution to yield the above tertiary amine as a colourless liquid b.p. 121°/12 mm. $[n]_D^{20°}$ 1.5062. (Watanabe (1949) gives b.p. 138°–155°/25.5 mm.) (Found : C, 74.2; H, 9.45; N, 8.02. $C_{11}H_{17}ON$ requires C, 73.7; H, 9.56; N, 7.84%.) The hydrobromide had m.p. 134°.

Other compounds used were made by methods described in the literature.

Summary

1. A biological assay method of determining the activity of amine oxidase inhibitors *in vitro* is described.

2. Nuclear-substituted choline phenyl ethers, and related compounds, inhibit the enzymic destruction of adrenaline by guinea-pig liver.

3. The more active nuclear-substituted choline phenyl ethers—such as choline *p*-tolyl ether, choline *o*-chlorophenyl ether, and choline *o*-tolyl ether—are, weight for weight, three times more active as inhibitors of amine oxidase than amphetamine, thirty times more active than ephedrine, and three hundred times more active than cocaine.

4. Among those compounds studied, the choline phenyl ether structure imparts maximum amine-oxidase-inhibitory activity.

5. In the choline phenyl ether series, *ortho* or *para* nuclear substitution increases amine-oxidase-inhibitory activity and *meta* substitution decreases it.

6. The inhibition of amine oxidase by choline phenyl ethers is competitive.

7. The relative activity of amphetamine and a choline phenyl ether inhibitor, and the competitive nature of the inhibition by the latter, have been confirmed by a manometric method.

8. The preparation and characteristics of several new compounds used in this study are recorded.

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THE ESTIMATION OF 5-HYDROXYTRYPTAMINE IN THE PRESENCE OF ADRENALINE

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Recently Amin, Crawford, and Gaddum (1954) developed a method for the extraction and estimation of 5-hydroxytryptamine (HT) which depended for assay on responses evoked in the isolated oestrous uterus of the rat. This preparation is sensitive to inhibition by adrenaline and noradrenaline, and it was appreciated that in some tissues there could be sufficient of these to make the HT estimate too low. The present study aimed at eliminating errors due to either adrenaline or noradrenaline in the estimation of HT in various tissues of the rabbit.

An attempt was made to separate HT from adrenaline by paper chromatography, elute the HT from the appropriate strip, and estimate the activity on the oestrous uterus of the rat—but a satisfactory method was not developed. Later, assays were based on vasoconstriction in the isolated perfused ear of the rabbit, where the aim was to minimize the effects of adrenaline and noradrenaline by the use of specific antagonists. But this method was very tedious, and was abandoned.

It was found that the inhibitory effect of adrenaline could be eliminated by the use of the polyphenoloxidase enzyme present in mushroom juice. So all the tissue extracts were treated with a standard preparation of crude mushroom juice before they were assayed on the fairly regular and sensitive isolated atropinized oestrous uterus of the rat.

METHODS

The Isolated Rat Uterus—Method for Assay.—Erspamer first used the atropinized oestrous uterus of the rat for the assay of enteramine, or HT (Erspamer, 1942, 1952). The method of Amin, Crawford, and Gaddum (1954) for the preparation of the uterus in oestrus and its use for the assay of HT was employed here. Doses of 5–20 ng. HT (in a 2 ml. bath) gave suitable responses, magnified 10–12 times, with a discrimination of ± 1–2 ng. for the estimations. A sample of 5-hydroxytryptamine creatinine sulphate was supplied through the courtesy of Upjohn Laboratories, Kalamazoo, Michigan.

At the end of each series of assays the rat uterus preparation was treated with lysergic acid diethylamide (LSD). This caused specific blockade of the response to HT (Gaddum, 1953). The effect of the tissue extracts was again tested on the uterus.

Sampling of the Rabbit Tissues.—Since clotted rabbit blood has a high HT equivalent (Erspamer, 1954a, b), efforts were made to remove as much blood as possible from the tissues before sampling. A rabbit was anaesthetized with ether, and its aorta was cannulated; perfusion of about 1 l. of isotonic saline was made at about 60 cm. water pressure. Blood and perfusate were allowed to escape through an opening in the inferior vena cava.

When samples from the head and neck were required the carotid arteries were cannulated and the perfusate allowed to escape by the jugular veins. The pulmonary artery was cannulated when lung samples were required. In one case where this was done a series of samples of perfusate was collected throughout the perfusion. They were later calibrated colorimetrically in terms of the first sample. This showed that when the tissue was creamy-white and appeared bloodless, and the perfusate was almost colourless, the latter contained less than 1% of blood. No oedema was apparent.

When the perfusate was almost colourless, samples of tissue were taken, weighed, and extracted. If there was any delay in the dissection, e.g., while the skull was being opened for the brain, any other tissue samples already collected were stored at −17° C.

On two occasions, after the perfusion was complete, electrodes were placed on the spleen, and stimulation was applied until the organ was quite contracted. It was then removed, weighed, and extracted.

In sampling the gut mucosae the whole stomach and intestines were removed and separated into stomach, upper half of the small intestine, lower half of the small intestine, and large intestine. Each portion was slit open throughout its length and washed under running tap-water. When clean it was blotted dry with filter paper. The whole mucosa of each area was scraped gently off with a blunt scalpel, weighed, and extracted; then an aliquot of the acetone extract was taken.

Method of Extraction of Tissue Samples.—Amin (1953) and Amin *et al.* (1954) have studied the quantitative extraction of HT from horse intestine. They found that the best results were obtained with a double

extraction of the tissues with acetone—the first extraction with 20 ml. A.R. acetone/g. tissue, and the second with 20 ml. 95% acetone/g. tissue.

This removed all the extractable HT in solution in the acetone and left the substance P in the insoluble residue.

In detail the use of this method here was as follows. The samples, on removal from the rabbit, were placed in weighing bottles, or the larger ones on watch glasses, and weighed. The latter were then removed to beakers. Twenty volumes of A.R. acetone was added to each, and the tissue finely divided with scissors, and pressed and stirred occasionally with a flattened glass rod for 1 hr. Tough or dense tissues, such as skeletal muscle, diaphragm, and heart, were cut up under the acetone and then rubbed in a mortar with silver sand. The supernatant fluid was filtered through a Whatman No. 1 paper, which had been washed with 5 ml. 95% acetone. When filtration was complete the papers were each washed with 2 ml. 95% acetone. To each of the residues was added 20 volumes of 95% acetone; these were again stirred and pressed for half an hour. The supernatant fluids were filtered, each through the same paper as before. The bottle, or beaker, and the precipitate were washed twice with 5 ml. 95% acetone, and the combined acetone filtrates and washings were evaporated to dryness, under reduced pressure at 30–35° C., 2 ml. ethanol being added when necessary to reduce frothing. One ml. of water was added to each residue, and the solution was extracted twice with 10–15 ml. of light petroleum (b.p. 40–60°) to remove lipids. The aqueous residue was evaporated to dryness under reduced pressure at 30–35° C. The extracts were stored overnight at −17° C. and assayed on the next day.

Samples of blood, obtained from rabbits under ether or urethane anaesthesia, were extracted with 20 volumes of A.R. acetone; after having been thoroughly shaken, the flasks were stored at 5° C. for half an hour. The extracts were filtered through acetone-washed papers, and the flasks and precipitates washed with 95% acetone as before. Extraction of the residues with 95% acetone was considered unnecessary. The evaporation and removal of the lipids was done as with the other samples. Serum, which was centrifuged from blood which had been allowed to clot at room temperature, was similarly extracted.

Preparation of the Mushroom Juice.—The acetone extracts of tissues are likely to contain some adrenaline or noradrenaline, which may interfere with the assay of their HT content. Adrenaline and noradrenaline are ortho-dihydroxy compounds, and HT has a mono-hydroxy structure. Thus it was thought that an enzyme preparation which specifically oxidized dihydroxy compounds could be used for the routine treatment of the extracts before their assay.

Keilin and Mann (1938) detailed the preparation and purification of a polyphenoloxidase from edible mushrooms, and investigated its nature and properties. They stated that even a crude extract of mushrooms oxidized with great velocity, mainly ortho-dihydroxyphenols, and but few monophenols. This difference in oxidation rate was largely due to a protracted lag period. The polyphenoloxidase, which is known also as tyrosinase (Sumner and Myrbäck, 1951; Sumner and Somers, 1953), occurs in bacteria, potatoes, and mushrooms, as well as in human skin and melanomata. It is an aerobic oxidase, containing copper, which converts catechol to *o*-quinone as the first step in the formation of melanin.

In the present work, edible mushrooms (*Psalliota campestris*) were chosen as a convenient source of the enzyme; they are easily available, and are free from the large quantities of starch which are present in the cheaper source, potatoes. 120 g. of fresh mushrooms were sliced and then ground in a household mincer. The pulp was rubbed in a mortar with about 10 g. of silver sand for 20 min. The juice was squeezed out through linen by hand. This yielded 80 ml. of a dark brown turbid fluid, which was slightly frothy. After storage at 5° C. overnight (reported by Keilin and Mann (1938) often to cause an increase in activity), it was distributed as 0.5 ml. samples in small test-tubes. These samples were freeze-dried by reduced pressure in a centrifuge over phosphorus pentoxide.

When the juice was completely dry the tubes were sealed and stored at 5° C. The residue was like a solid foam and was brown in colour. This redissolved readily to give a slightly turbid solution, which was diluted to 5 ml., and 0.25 ml. of this, "the enzyme preparation," was added to every 1.0 ml. of tissue extract.

Tests of enzyme activity were always based on the time taken for a given amount of enzyme preparation to eliminate the inhibitory effect of a certain amount of adrenaline, or noradrenaline, on the spasm of rat oestrous uterus caused by HT. For the treatment of tissue extracts some of the enzyme preparation was added to the aqueous solution of the acetone extract residue, and these were allowed to stand together at room temperature for at least 40 min., then an assay of the HT activity was made in the usual way. The *p*H of the samples was always between 4.5 and 6.0.

Results

The Action of the Mushroom Juice.—In the experiment shown in Fig. 1, regular responses to 10 ng. of HT were recorded. Each dose was left in contact with the tissue for 45 sec., and 4 min. elapsed between doses. At C, 10 ng. of HT was again given, but within the same dose volume there was also 10 ng. of adrenaline. The presence of the adrenaline caused inhibition of the response, and the inhibitory effect continued and gradually disappeared as the following responses to 10 ng. HT (alone) were recorded.

The solution M contained 100 ng. HT, 100 ng. adrenaline and 0.20 ml. of the enzyme preparation in each ml. The first dose of M, containing 10 ng. of each drug, was given 2 min. after the addition

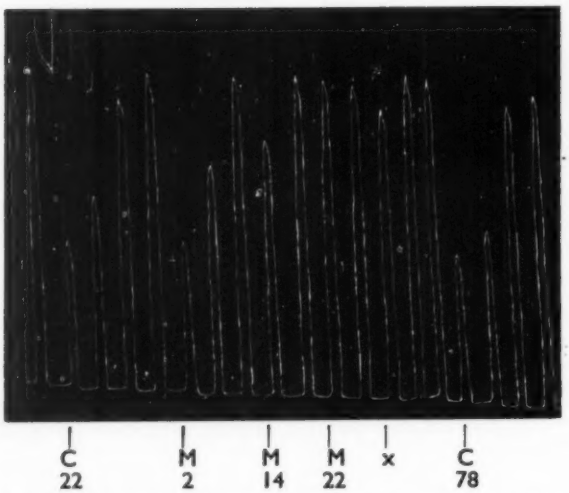

FIG. 1.—Rat oestrous uterus in 2 ml. bath. Responses to 10 ng. HT. At C, 10 ng. HT + 10 ng. adrenaline. At M, 10 ng. HT + 10 ng. adrenaline + mushroom juice. Numerals below C and M indicate time in min. from preparation of mixture. At x, 8 ng. HT.

of the enzyme preparation, and the inhibition and recovery of the responses was similar to that recorded for the dose C. Then another dose of M, now 14 min. from its preparation, was given. This caused a less inhibited response, and no inhibition of the following doses of 10 ng. HT. At 22 min. from preparation, M did not show any inhibitory effect, whereas the action of the sample without enzyme was as before (see C at 78 min.).

Thus, by standing at room temperature, the enzyme preparation was able to abolish in 22 min. the inhibitory effect of 10 ng. adrenaline on the uterine contraction caused by 10 ng. HT. It was found also that the enzyme had no effect on the responses to HT alone, unless the preparation and the drug were allowed to be in contact for more than 80 min., when the HT response became slightly reduced.

The results of further experiments showed that the inhibitory effect of adrenaline, when present in concentrations up to ten times that of the HT, could be abolished always within 40 min. from addition of the enzyme preparation. Under similar conditions, noradrenaline in concentrations up to one hundred times that of the HT was also inactivated.

Estimates of HT in the Tissues of Rabbits.—Using the acetone extraction method for the samples of tissues, and the treatment of the extracts with the enzyme preparation for at least 40 min., and not more than 80 min., the estimates of the HT activity were made by assay on the atropinized oestrous uterus of the rat. In most cases, analyses of tissues from three or more animals were carried out, in order to obtain some indication of the extent of the individual variation.

The results are given in Table I.

TABLE I

HT EQUIVALENT IN μG./G. RABBIT TISSUE

(Where extract activity was not abolished by LSD, estimates are given in parentheses)

Sample	Individual Estimates	Mean
Spleen:		
Unstimulated	21·0, 13·8, 11·1, 9·1, 7·7, 7·6	11·7
Stimulated	13·3, 9·1	11·2
Mucosae:		
Stomach	7·5, 6·4, 4·6	6·2
Upper small intestine	4·0, 1·4, 1·2	2·2
Lower small intestine	3·7, 0·67, 0·52	1·6
Large intestine	4·0, 3·5, 3·0	3·5
Brain:		
Hypothalamus	0·42, 0·40, 0·36	0·39
Mid-brain	0·50, (0·49), 0·13	0·31
Olfactory bulbs	0·21, 0·14, (0·04)	0·17
Cerebellum	(0·05), 0·04, 0·03	0·035
Liver:		
Without portal perfusion	1·45, 0·90, 0·48	0·94
With " "	(0·44), (0·25), (0·13)	(0·27)
Bone marrow	0·39, 0·30, 0·29	0·33
Lung:		
With direct pulmonary perfusion	(0·32), 0·23, 0·16, 0·12, 0·03	0·13
Without direct pulmonary perfusion	0·30, 0·16, 0·07	0·18
Thyroid	(0·23), 0·09	0·09
Pancreas	0·21, (0·08), 0·05	0·13
Diaphragm	0·18, 0·12, 0·04, <0·02	0·09
Salivary glands	0·13, 0·07, 0·06, <0·03	0·07
Thymus	0·05, 0·05	0·05
Heart	0·04, 0·03, <0·02	0·023
Bladder	0·13, 0·11, 0·03	0·09
Kidney	0·15, 0·10, 0·02	0·09
Skeletal muscle	Not detected	
Sciatic nerve	" "	
Adrenal glands	" "	

One series of rabbit tissue extracts was assayed both before and after treatment with the enzyme preparation. The increases in the HT equivalents are shown in Table II. No increase was found with serum—normal serum is unlikely to contain significant amounts of adrenaline or noradrenaline. In this experiment none of the extracts gave any response after the uterus preparation was treated

TABLE II

HT EQUIVALENTS (μG./G.) OF ACETONE EXTRACTS OF RABBIT TISSUES BEFORE AND AFTER TREATMENT WITH MUSHROOM JUICE

Tissue	Before	After	Ratio
Kidney	0·020	0·025	1·25
Lung	0·060	0·075	1·25
Pancreas	0·145	0·210	1·45
Liver	0·560	0·90	1·6
Serum	4·40	4·40	1·0

with lysergic acid diethylamide, so the increase is probably due to an unmasking of more HT activity.

Discussion

At the beginning of this study on the HT content of acetone extracts of rabbit tissues, it was appreciated that some, at least, of the extracts might contain sufficient adrenaline to cause the estimates on the isolated rat uterus to be too low. This effect was abolished by the use of the polyphenoloxidase present in mushrooms.

Mushroom Polyphenoloxidase.—The enzyme in crude mushroom juice proved very suitable for the destruction of the inhibitory activity of adrenaline on the rat uterus in mixtures of adrenaline and HT. The enzyme preparation was easy to use, since it is active at *p*H 4–6, which was the reaction of the extracts, and since it is active at room temperature.

The juice contains both polyphenoloxidase and monophenoloxidase. Fortunately for my purpose, the lag time before the monophenoloxidase had its maximum action was such that the adrenaline present was oxidized by the polyphenoloxidase before the monophenoloxidase attacked the HT. By measurement of the oxygen uptake and the loss of biological activity, Philpot (1940) showed a conversion of over 95% of adrenaline to adrenochrome by a preparation of polyphenoloxidase from mushrooms.

The HT Equivalent of Rabbit Tissues.—The HT equivalent for rabbit blood is high, compared with that for other animals (Erspamer and Faustini, 1953 ; Erspamer, 1954a, b), so that the blood had to be removed, so far as possible, from the tissues before sampling. This was done with an isotonic saline perfusion, and was complete in about 15 or 20 min. The perfusion may also have washed some HT out of the tissues themselves, but this is thought unlikely. Another danger, possibly incurred by this perfusion treatment, is that of causing oedema of the tissues, in which event the HT equivalent determined per gram of sample would be low. There was no evidence of oedema to naked eye inspection, except in one sample of thyroid and one of submaxillary glands, which were rejected. The lung tissue invariably floated in the acetone.

When the perfusate was almost colourless it contained less than 1% of blood. Thus if a 2 g. sample of tissue were to contain 1 ml. of this perfusate, the " blood HT " content of this sample would not be more than 0.025 μg. (rabbit blood contains 2.0–2.5 μg. HT/ml.). The tissue HT equivalents were considered to be significant when they were more than 0.1 μg./g.

The estimate of HT in rabbit blood was 2.0–2.5 μg./ml. whole blood and 4.4 μg./ml. serum, from acetone extracts in each case. The HT equivalent given by Erspamer and Faustini (1953) for rabbit serum is 3.53 μg./ml. Another result is given by Erspamer and Sala (1954): it is 4.3 μg./ml. serum, from ten samples. Humphrey and Jaques (1954) give a value of 5.2 μg. HT/ml. whole blood which is high compared with these other estimates. Using a chemical method involving the fluorimetric estimation of HT in an extract of platelets, Udenfriend and Weissbach (1954) find 4.0 μg. HT/ml. whole blood.

A high HT equivalent was found for the spleen, though the mean estimate was lower than that found by Erspamer. This discrepancy is possibly due to the blood content of the spleens sampled by Erspamer (1954a, b). In the present work the contraction of the spleen caused by electrical stimulation and leading to the expulsion of perfusion fluid from the sinuses caused no significant difference in its HT equivalent.

The values for HT in the mucosae of the various areas of the intestinal tract correspond approximately to those of Erspamer (1954a and b).

Using the atropinized rat colon for assays, Feldberg and Toh (1953) obtained higher HT equivalents for acetone extracts of rabbit gastric mucosa ; fundic mucosa yielded 7.5–10 μg. HT/g. and pyloric mucosa 1.25 μg. HT/g. Yet the relatively high HT content throughout these samples of mucosa is consistent with their high content of enterochromaffin (argentaffin) cells.

The bone marrow had a fairly constant significant content of HT, but it would be difficult to say whether this was due entirely to that in the platelets of the blood passing through the vascular sinuses, or whether part of it was due to HT in the megacaryocytes (already synthesized for the time when these cells break down to form platelets).

When the liver was perfused with isotonic saline, through the portal vein as well as through the aorta, the HT equivalent of the samples was lower than when perfusion was through the aorta only. The most likely reason for this difference is that in the second case some blood was held in the portal sinuses. A volume of 0.25 ml. blood per gram of the tissue would contribute 0.5 μg. HT/g. sample. The proportion, 1 to 3, of portal blood to liver tissue is perhaps rather high, but it may be that, though less blood is present, excess of platelets is trapped in the tissue spaces before

destruction by the liver cells, and that these platelets, as well as the blood, are washed out by the portal perfusion.

Other tissues which contained small but significant amounts of HT (more than 0.1 μg./g.) were the hypothalamus, thyroid, and pancreas. It is interesting to note that Erspamer (1937) described enterochromaffin cells in the pancreas of certain mammals, including the hare, but that of the rabbit is not mentioned.

The effect of HT on the atropinized oestrous uterus of the rat is specifically blocked by LSD (Gaddum, 1953). After each assay sufficient LSD was added to the organ bath to abolish the response to the standard HT. With extracts of all the tissues mentioned above—blood, serum, spleen, mucosae, bone marrow, liver, lung, hypothalamus, thyroid and pancreas (except the "portal perfused" liver)—the activity was abolished by LSD. Thus, in these extracts, at least a very large proportion of the activity was probably due to HT. The tests on the liver samples, positive when the HT-equivalent was high and negative when it was low, suggest that there is some other uterus-contracting substance in these extracts.

In the extracts of each of the other tissues sampled, the activity was neither consistently abolished by LSD nor consistently resistant to this antagonist. The mean estimate for each of these tissues was never more than 0.1 μg. HT/g. It may be said that there was a trace of HT in the diaphragm, salivary glands and thymus, but in the heart, bladder, kidney, skeletal muscle and sciatic nerve no definite evidence for the presence of HT was obtained.

No uterus-stimulating activity was recorded from the adrenal extracts, but only an inhibitory effect on subsequent responses to HT. This observation results presumably from the incomplete destruction of the adrenaline by the enzyme preparation. The extracts became faintly red in colour, because of the relatively high concentration of adrenochrome.

Summary

1. A survey of the HT content of acetone extracts of the various tissues of the rabbit has been made; the atropinized oestrous uterus of the rat was used for the assays.

2. The possible interference by adrenaline in the estimations was eliminated by treatment of the extracts with mushroom juice, which contains a polyphenoloxidase enzyme.

3. Significant amounts of HT were found in the spleen, various areas of the gut mucosa, blood and serum. The hypothalamus, the liver, and the bone marrow showed smaller amounts of HT. Other tissues (lung, thyroid, pancreas, and diaphragm) contained little HT, while in yet others HT was not detected (skeletal muscle, nerve, and adrenals).

4. The only test of specificity of the extract activity was by the effect of the extract on the LSD-treated uterus. LSD abolished the response, as it did that to HT, to active extracts of those tissues mentioned above, but not to extracts of mid-brain, olfactory bulbs, cerebellum and the tissues containing only traces of activity. These tissues must contain some other active substance.

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THE ACTION OF SOME TRYPANOCIDAL AND ANTIMALARIAL COMPOUNDS ON *BABESIA RODHAINI (PIROPLASMIDEA)*

BY

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This paper describes an investigation into the effects of a series of well-known trypanocidal and antimalarial compounds on the course of infection with the piroplasm *Babesia rodhaini* in white mice.

B. rodhaini was isolated from the blood of the rodent *Thamnomys surdaster surdaster* by van den Berghe, Vincke, Chardome, and van den Bulke (1950). The organism was transmitted to white mice and is now maintained in these animals. *B. rodhaini* promised to be a useful organism for the screening of drugs designed for the treatment of piroplasmosis; the results of some investigations in this field have already been published (Rodhain, 1951; Beveridge, 1953).

During the course of other work with *B. rodhaini* in this laboratory, it was discovered that infections could be cured with the trypanocidal compounds antrycide and 1-methyl-l-phenyldithiobiuret (Godfrey, 1955), whereas the antimalarial drugs mepacrine and proguanil were without effect. This result is surprising when it is considered that the *Piroplasmidea* are much more nearly related to the *Haemosporidiidea* than they are to the *Trypanosomidae*. Accordingly, it was decided to investigate the action upon *B. rodhaini* of a series of compounds which were known to have either trypanocidal or plasmodicidal properties.

METHODS

The strain of *B. rodhaini* used was the Antwerp strain, obtained through the courtesy of Miss E. Beveridge, of the Wellcome Laboratories of Tropical Medicine. The method of testing the drugs against *B. rodhaini* was adapted from the standard screening procedure used in our laboratories. White mice were inoculated intraperitoneally with three million parasitized erythrocytes contained in 0.2 ml. of citrated saline, and were then treated daily with the drug for four days, the first dose being given intraperitoneally four hours after infection. Thin blood films were made on the sixth day, stained with Giemsa, and the percentages of parasitized erythrocytes were recorded. The control animals generally showed a parasitaemia of 70–80% at this time and they usually died from the infection on or about the eighth day. The blood of mice which survived for eight days or longer was examined at weekly intervals for a further four weeks in order to follow the course of infection.

The drugs used were (*a*) antimalarial compounds: chloroquine diphosphate; mepacrine hydrochloride; pamaquin naphthoate; proguanil acetate; pyrimethamine; (*b*) trypanocidal compounds: antrycide methyl sulphate; "Berenil," di-(4-amidinophenyl)-triazene-(*N*-1:3) diaceturate-3H_2O (Milne, Robson, and Lwebandiza, 1955; Bauer, 1955; Enigk and Reusse, 1955); B314, 2-*p*-acetaminostyryl-6-methylaminoquinoline methosulphate (Browning, Cohen, Ellingworth, and Galbransen, 1929); "Congasin," bis-2-methyl-4-aminoquinolyl-6-melamine; ethidium bromide; RD1660, 1-methyl-1-phenyl dithiobiuret (Woolfe, 1953); the antibiotic "Stylomycin" (Porter *et al.*, 1952); suramin; tartar emetic; tryparsamide. All drugs were dissolved in sterile saline or sterile distilled water. The chronic toxicity of each drug was estimated by intraperitoneal injection into mice daily for four days. The maximum tolerated dose (M.T.D.) was taken as being the lower of two doses, one twice the other, of which the upper killed but the lower did not. Two mice were treated at each dose level. Six mice infected with *B. rodhaini* were treated with each drug at its M.T.D. When a drug was active, further batches of mice were treated at fractions of this dose. The minimum effective dose was taken to be the dose which limited the mean parasitaemia in the treated mice to 5% of the mean parasitaemia in the controls on the sixth day after infection. A drug was considered to have cured the infection when no parasites were seen in blood films from treated animals taken once weekly for four weeks after treatment.

RESULTS

The results of the tests carried out on five antimalarial and ten trypanocidal compounds are

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summarized in Table I. The antimalarial compounds were all without action, but six of the ten trypanocidal compounds showed activity. Five compounds, antrycide methyl sulphate, ethidium bromide, RD1660, "Stylomycin," and "Berenil,"

TABLE I

THE ACTION OF SOME TRYPANOCIDAL AND ANTIMALARIAL COMPOUNDS ON *B. RODHAINI*

Compound	Type	Max. Tolerated Dose mg./kg. Daily×4	Min. Effective Dose mg./kg. Daily×4	Curative Action
Chloroquine ..	Antimalarial	50	>50	−
Mepacrine ..	,,	100	>100	−
Pamaquin ..	,,	50	>50	−
Proguanil ..	,,	10	>10	−
Pyrimethamine	,,	350	>350	−
Antrycide ..	Trypanocidal	10	3·5	+
B314	,,	50	50	−
Berenil	,,	50	5·0	+
Congasin ..	,,	75	>75	−
Ethidium bromide	,,	20	3·0	+
RD1660 ..	,,	500	40	+
Stylomycin ..	,,	150	90	+
Suramin ..	,,	100	>100	−
Tartar emetic ..	,,	10	>10	−
Tryparsamide ..	,,	2,500	>2,500	−

cured the infection in all mice which were treated at the maximum tolerated dose. At lower doses they considerably delayed the progress of the parasitaemia but did not prevent the infection from killing some of the mice. The sixth compound which showed activity, B314, delayed the parasitaemia, but some of the mice died even when treated at the maximum tolerated dose. The remaining trypanocidal compounds, suramin, "Congasin," tartar emetic, and tryparsamide, were all without effect.

DISCUSSION

These results show that six of the ten trypanocidal compounds which were tested affected the course of infections of *B. rodhaini* in white mice; five antimalarial compounds were all without action. In a previous investigation, Beveridge (1953) found that the aromatic diamidines, stilbamidine, phenamidine, and pentamidine and 2:8-diamine-10-methylacridinium, a constituent of acriflavine, were all active against *B. rodhaini*. Thus *B. rodhaini* seems to be susceptible to drugs which also act against trypanosomes—especially *T. congolense*—but it is resistant to antimalarial compounds. These findings are in accordance with the views expressed by Goodwin and Rollo (1955). Congasin is an exception in that it is very active against *T. congolense* but does not affect *B. rodhaini*.

The exact systematic position of the *Piroplasmidea* is still a matter of some controversy, but it is generally agreed that they are fairly closely related to the *Haemosporidiidea*. It is therefore surprising to find that from the viewpoint of chemotherapy the affinities of *B. rodhaini* are with trypanosomes rather than with malarial parasites. In this respect it is interesting to recall that several attempts have been made to relate the piroplasms to the haemoflagellates, notably that of Léger and Dubosq (1910).

The trypanocidal compounds which are active against *B. rodhaini* are of widely differing structure and it seems unlikely that all these compounds affect any single enzyme system common to both *B. rodhaini* and trypanosomes. It is more reasonable to suppose that there is a closer resemblance between the metabolic systems of the parasites than might be expected from their different morphology and habits.

The results of these investigations indicate that trypanocidal compounds may prove to be useful agents in the chemotherapy of other piroplasmoses. According to recent reports, the trypanocidal compound "Berenil" has been successfully used in the treatment of bovine, ovine, and canine piroplasmoses in the field (Bauer, 1955; Enigk and Reusse, 1955).

SUMMARY

1. The following trypanocidal compounds were able to cure white mice infected with *Babesia rodhaini*: antrycide methyl sulphate, ethidium bromide, "Berenil," the dithiobiuret compound RD1660, and the antibiotic "Stylomycin."

2. A styryl quinoline compound (B314) delayed the progress of the infection but did not cure all mice treated at the maximum tolerated dose.

3. Four other trypanocidal compounds, "Congasin," suramin, tartar emetic, and tryparsamide, were without effect.

4. Five antimalarial compounds, chloroquine diphosphate, mepacrine hydrochloride, pamaquin naphthoate, proguanil acetate, and pyrimethamine, were inactive.

5. In many respects the chemotherapeutic reactions of *B. rodhaini* resemble those of trypanosomes, especially *T. congolense*, and are unlike those of malarial parasites.

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Drug Co., Ltd., for the supplies of ethidium bromide and RD 1660, to Farbwerke Hoechst, Ag., for supplies of " Congasin " and " Berenil," and to Lederle Laboratories, Ltd., for supplies of " Stylomycin."

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THE ACTION OF 5-HYDROXYTRYPTAMINE ON THE NICTITATING MEMBRANE AND ON THE SUPERIOR CERVICAL GANGLION OF THE CAT

BY

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An action of 5-hydroxytryptamine (5-HT) on the nictitating membrane has been described by several workers. Erspamer (1954) observed that in spinal cats the intravenous injection of 5-HT caused a larger contraction of the previously denervated nictitating membrane than of the normal. Reid and Rand (1952) observed that a contraction of the normal nictitating membrane was caused by injection of small amounts of 5-HT into the carotid artery. This suggested a direct action of 5-HT on the membrane, which was confirmed by Thompson (1955) in his experiments with the isolated nictitating membrane. Observations by Lecomte (1955) furthermore showed that the adrenal glands did not play an important part in the response of the nictitating membrane to intravenously injected 5-HT.

The first suggestion that 5-HT acted on autonomic ganglia was made by Gaddum and Hameed (1954), who obtained evidence for the view that this substance acted on tryptamine receptors present in the nervous tissues of the ileum of the guinea-pig. Robertson (1953, 1954a) similarly obtained evidence for such an indirect action of 5-HT on the guinea-pig ileum, though he interpreted it as an action on the nerve endings. Robertson (1954a) also observed that 5-HT caused stimulation of the perfused superior cervical ganglion; this stimulating action was not blocked by hexamethonium (1954b).

The present work was undertaken in order to obtain more information about the action of 5-HT on the superior cervical ganglion and on its effector organ, the nictitating membrane.

METHODS

Observations of the action of 5-HT on the normal and denervated nictitating membrane were made in spinal preparations 7–14 days after excision of the right superior cervical ganglion.

Perfusions of the heads of cats were made according to the procedure described by Burn and Trendelenburg (1954). This consists in principle in perfusing the head with a warmed mixture of 1 part of a 6% dextran solution and 3 parts of Locke solution through the carotid arteries, the outflow being collected from the external jugular veins. Injections were made into the perfusion fluid just before it reached the Y-piece, so that the injected material was evenly distributed to both sides.

For intra-arterial injections cats of 2–4 kg. were used. After inducing anaesthesia with ether, 80 mg./kg. chloralose was injected intravenously. For intra-arterial injections the lingual artery was tied and a cannula inserted pointing centrally. Injections were made when the external and internal carotid arteries were either occluded or left open. For the sake of brevity the first mode of injection will be described as "injection to the ganglion," the latter as "injection to the nictitating membrane," as earlier observations showed that an intra-arterial injection during occlusion of the external carotid artery acted on the superior cervical ganglion and not on the nictitating membrane (Trendelenburg, 1954).

Contractions of the nictitating membrane were recorded by attaching them to isotonic levers fitted with frontal writing points; the contractions were magnified 7.5 times.

The cervical sympathetic chain was always divided. When electrical stimulation was necessary the peripheral end of the cervical sympathetic chain was placed on shielded electrodes and covered with warm liquid paraffin. Electrical stimuli of 0.7 msec. duration were applied at a rate of 2 or 15/sec.

For intra-arterial injections carefully neutralized solutions were used, the injected volume never exceeding 0.2 ml. The following substances were used: 5-hydroxytryptamine creatinine sulphate, nicotine hydrogen tartrate, hexamethonium bromide, atropine sulphate, mepyramine maleate, cocaine hydrochloride. All doses were expressed as salts.

Results

Intact Spinal Cat.—One to two weeks after excision of the superior cervical ganglion of the right side the response of the normal and of the previously denervated nictitating membrane to the intravenous injection of 5-hydroxytryptamine (5-HT) was determined. The ganglion of the normal side was left intact, but the preganglionic fibres were cut. Results obtained in 7 spinal preparations showed clearly that the injection of 10 μg. 5-HT caused a much stronger contraction of the denervated than of the normal side, the ratio D/N being 3.44. After the intravenous injection of 30 μg. 5-HT the response of both membranes was increased, but the ratio fell to 1.13. After injection of 100 and 300 μg. 5-HT, however, the contraction of the normal side was usually greater than that of the denervated membrane, the mean ratios being 0.89 and 0.78 respectively.

Perfusion of the Head of the Cat.—In spinal preparations factors other than the direct action of a substance on the nictitating membrane may influence the response of this smooth muscle. The action of 5-HT was therefore studied in the perfused head of the cat. The injection of 10–100 μg. 5-HT into the perfusion fluid was found to cause a contraction of the normal nictitating membrane in 6 preparations, failing only in one experiment in which the injection was made very late. In three preparations the response of the previously denervated membrane to 5-HT was also determined, and it was found that it responded much more strongly than the normal membrane. This is shown in Fig. 1, where the response to the injection of 40 μg. 5-HT (HT) was compared with the response to 4 μg. noradrenaline (N), 1 mg. tyramine (T), and 10 μg. adrenaline (A). This example shows that 5-HT had a direct action on the nictitating membrane and that it resembled adrenaline rather than tyramine in its action, though it was less effective than adrenaline.

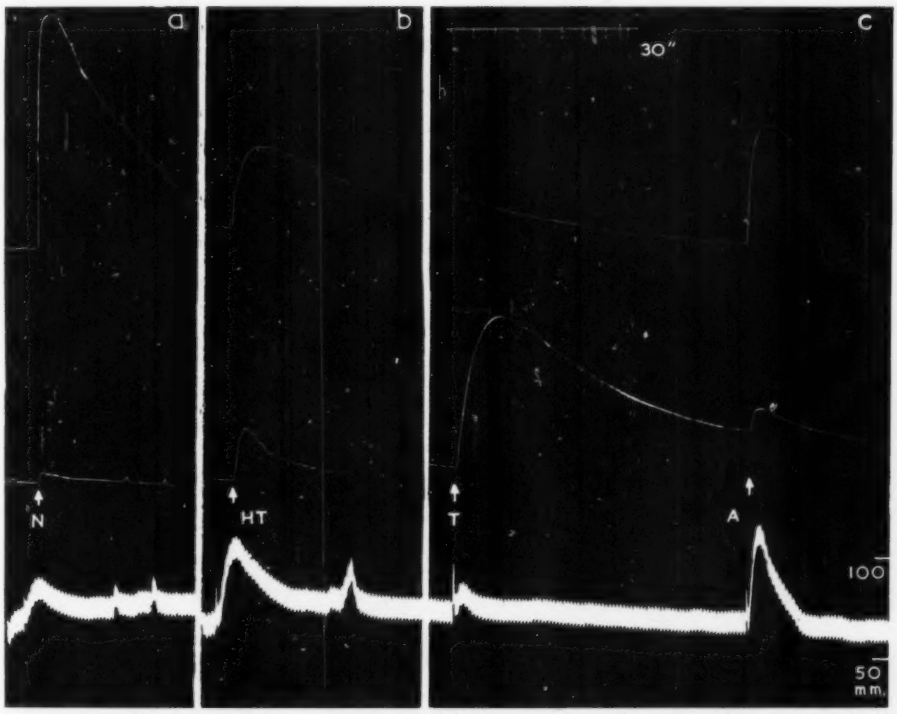

FIG. 1.—Perfused head of spinal cat. Denervated nictitating membrane (upper trace), normal nictitating membrane (middle trace) and perfusion pressure. Injection into the perfusion fluid of 4 μg. noradrenaline (N), 40 μg. 5-HT (HT), 1 mg. tyramine (T) and 10 μg. adrenaline (A). Interval of 3 min. between (a) and (b) and of 2 min. between (b) and (c).

Stimulation of the Superior Cervical Ganglion.—5-HT was injected intra-arterially through the central end of the lingual artery during occlusion of the internal and external carotid arteries (in the following described as "injection to the ganglion"). The dose ranged from 1 to 100 μg. in different experiments. This injection caused a contraction of the nictitating membrane of 17 out of 24 cats under chloralose anaesthesia (=70%).

In order to determine whether the contraction of the nictitating membrane observed after an injection of 5-HT to the ganglion was in fact due to stimulation of the ganglion, it was always compared with an injection of the same amount of 5-HT to the membrane (i.e., intra-arterial injection through the lingual artery when external and internal carotid arteries were left open). It was concluded that the response of the nictitating membrane to the injection to the ganglion was due to stimulation of the superior cervical ganglion for the following reasons:

(1) In sensitive preparations as little as 1–10 μg. 5-HT caused a contraction of the nictitating membrane when injected to the ganglion, but not when injected to the membrane.

(2) Extirpation of the ganglion abolished the response to the intra-arterial injection to the ganglion.

(3) When the sensitivity of the ganglion was low, larger amounts of 5-HT (10–100 μg.) had to be injected in order to obtain stimulation of the ganglion. These larger amounts had also a direct action on the nictitating membrane, making it more difficult to distinguish between a contraction in response to ganglionic stimulation and a contraction due to the direct action of 5-HT on the smooth muscle of the membrane. Fig. 3 shows, however, that the response to an injection to the ganglion (x) was usually of shorter duration than the response to an injection to the membrane (o).

(4) When large amounts of 5-HT had to be injected, nicotine helped to differentiate the sites of action. Fig. 2 shows the response of the nictitating membrane to the injection of 50 μg. 5-HT, first to the ganglion (x) and then to the membrane (o). This was repeated 1 min. after three successive injections of 100 μg. nicotine each, which blocked the action of 5-HT on the ganglion but did not depress its action on the nictitating membrane (Fig. 2b). Thirty min. later 5-HT stimulated the ganglion again (Fig. 2c).

(5) The adrenal gland did not play any role in the response of the nictitating membrane, since the membrane of the other side did not contract (Fig. 2) and since adrenalectomy failed to change the response.

The action of a ganglion-blocking substance of the competitive type (hexamethonium) is illustrated in Fig. 3. It shows the response of the nictitating membrane to the intra-arterial injection of 100 μg. 5-HT, first to the ganglion (x) and then to the membrane (o), demonstrating the difference in duration of the responses. Fig. 3b shows the repetition of the injections 3 min. after an intravenous injection of 5 mg. hexamethonium, the responses being identical with the earlier ones. In 11 experiments hexamethonium was injected either intravenously (5–20 mg.) or intra-arterially (0.5–2 mg.), and these injections were found to produce complete block of ganglionic transmission. The response of the ganglion to 5-HT was, however, unchanged in 7 experiments, increased in one (+28% of the initial contraction) and decreased in 3 experiments (−28, −28, −40%). Thus hexamethonium usually failed to block the stimulation of the superior cervical ganglion by 5-HT.

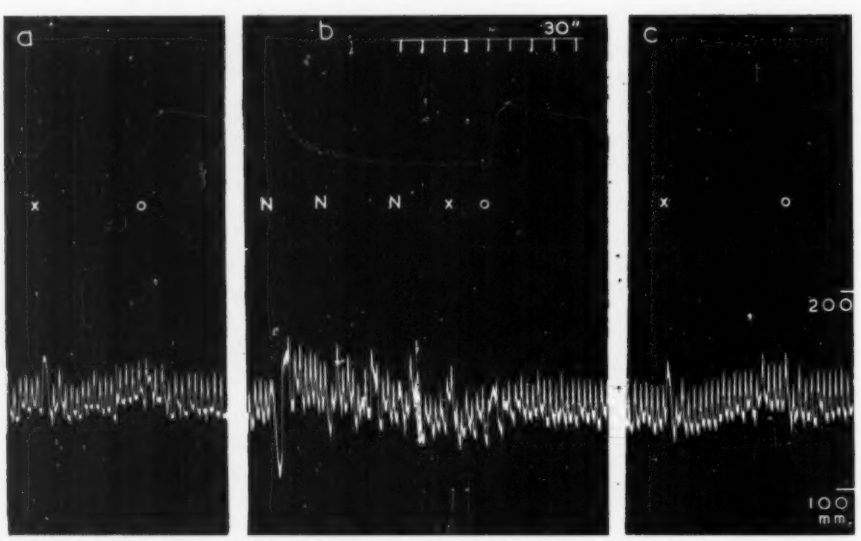

FIG. 2.—Cat under chloralose, 2.7 kg. Nictitating membrane of the injected side (top trace), of the other side (middle trace) (both membranes normal), and arterial blood pressure (bottom trace). Intra-arterial injections of 50 μg. 5-HT to the ganglion (x) and to the nictitating membrane (o), in (a) before, and in (b) immediately after, three intra-arterial injections each of 100 μg. nicotine (N). Record (c) was obtained 30 min. later.

A further differentiation between the actions of 5-HT on the ganglion and on the nictitating membrane was possible by the use of cocaine, which abolished or reduced the response of the ganglion to 5-HT when injected intravenously in amounts of 5–10 mg. Fig. 3c shows the response to the repetition of earlier injections

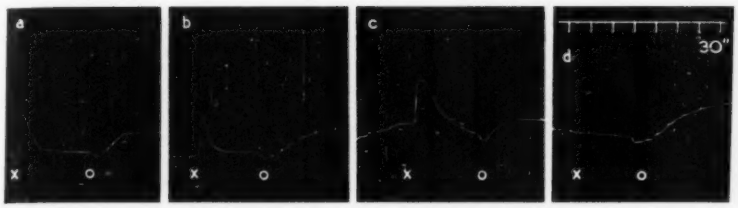

FIG. 3.—Cat under chloralose, 2.1 kg. Normal nictitating membrane. Intra-arterial injections of 100 μg. 5-HT to the ganglion (x) and to the nictitating membrane (o). 30 min. intervals between records; (b) was obtained 3 min. after 5 mg. hexamethonium, (c) 5 min. after 2 mg. cocaine, (d) 5 min. after 6 mg. cocaine, all injected intravenously.

of 5-HT 5 min. after the intravenous injection of 2 mg. cocaine. This amount of cocaine failed to modify the response, but after the intravenous injection of 6 mg. cocaine the response of the ganglion was abolished, while the direct action of 5-HT on the membrane was potentiated (Fig. 3d). The reduction of the action of 5-HT on the ganglion and the potentiation of its direct action on the membrane were observed regularly.

When atropine was injected in an amount which antagonized the response of the ganglion to pilocarpine, the response to an intra-arterial injection of 5-HT to the ganglion was found to be reduced, but such an amount of atropine was also found to reduce the response of the membrane to noradrenaline. The intravenous injection of 0.5 mg. mepyramine, on the other hand, did not affect the response of the ganglion to 5-HT, while abolishing that to histamine.

Desensitization to 5-HT by 5-HT itself was pronounced, necessitating time intervals of 10 to 30 min. between two injections of 5-HT, in order to obtain a similar response to the second injection. Before testing the action of the above-mentioned substances on the stimulation of the ganglion by 5-HT the right time interval was always determined.

Potentiation of Ganglionic Transmission.—As the results hitherto described suggested that 5-HT had ganglionic actions very similar to those of histamine and pilocarpine (Trendelenburg, 1954), its action on ganglionic transmission was investigated. When the preganglionic fibres of the ganglion were stimulated submaximally for 5 sec. twice per min. at a frequency of 2 shocks/sec., a series of contractions of the nictitating membrane was obtained as shown in Fig. 4 (b to g). The intra-arterial injection to the ganglion of 0.2 ml. saline solution and of 0.001 μg. 5-HT failed to change the response to electrical stimulation (Fig. 4b). When, however, increasing amounts of 5-HT were injected to the ganglion, potentiation of the response became more and more pronounced. When expressing the potentiation as % of the initial height of contraction, it was found to be 50% for 0.01 μg., 115% for 0.1 μg., 190% for 1 μg., 190% for 10 μg., and 213% for 100 μg. 5-HT. The response to the nictitating membrane did not significantly increase when the dose of 5-HT was increased from 1 to 10 and even to 100 μg., but comparison with the response to supramaximal stimulation (Fig. 4a) shows that the injection of 1 μg. 5-HT increased the response to nearly maximal height. The greater effect of

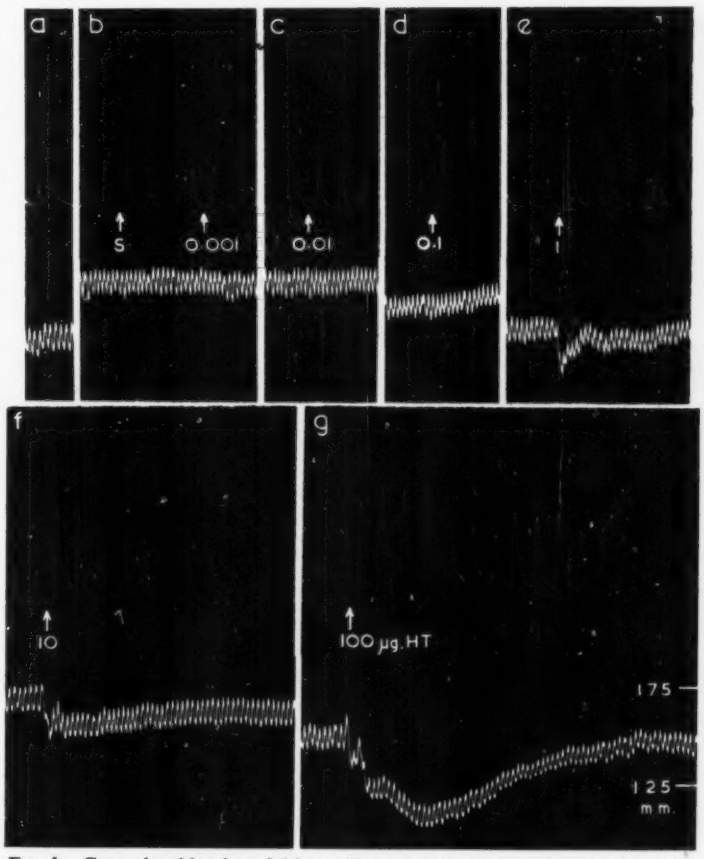

FIG. 4.—Cat under chloralose, 2.1 kg. Normal nictitating membrane and arterial blood pressure. Preganglionic stimulation for 5 sec. twice per min. at a rate of 2/sec. with supramaximal strength in (a) and submaximal strength in (b–g). Intra-arterial injections to the ganglion of 0.2 ml. saline and of 0.001 μg. 5-HT in (b), of 0.01 μg. in (c), 0.1 μg in (d), 1 μg. in (e), 10 μg. in (f), and 100 μg. 5-HT in (g).

increasing doses of 5-HT was, however, shown by a prolonged duration of the potentiating action. Fig. 4 also shows that the injection of 10 and 100 μg. 5-HT caused a contraction of the nictitating membrane. These larger doses therefore not only potentiated ganglionic transmission but also stimulated the ganglion cells themselves. Furthermore, in spite of the stimulation of the ganglion by a large dose of 5-HT there was no nicotine-like paralysis.

The response to supramaximal stimulation of the preganglionic fibres was not potentiated. Fig. 5 shows an experiment in which the intra-arterial injection of 10 μg. 5-HT potentiated the response to submaximal stimulation (a), failed to excite the unstimulated ganglion (b) and also failed to change the response to supramaximal stimulation (c). A potentiating action of 5-HT was also observed, when submaximal preganglionic stimulation was applied continuously instead of intermittently.

As the potentiation of the effect produced by submaximal preganglionic stimulation could have been caused by a lowering of the threshold of the nerve by 5-HT, the cervical sympathetic chain was split longitudinally in some experiments and a portion of the preganglionic fibres was stimulated supramaximally. This operation excluded any effect of 5-HT on the nerve threshold, but it provided a submaximal stimulus to the ganglion. A similar potentiation was observed under these conditions.

The action of atropine, mepyramine, and cocaine on the potentiation of ganglionic transmission by 5-HT is shown in Fig. 6. Throughout the experiment the same submaximal preganglionic stimulation and the same intra-arterial injection of 1 μg. 5-HT was used. The effect of 5-HT was not

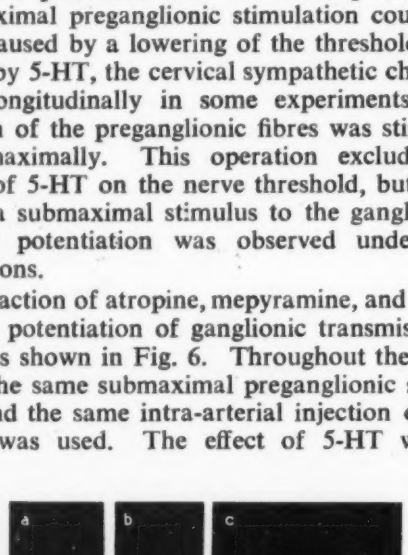

FIG. 5.—Cat under chloralose, 3 kg. Normal nictitating membrane. Intra-arterial injections to the ganglion of 10 μg. 5-HT at arrow, (a) during submaximal preganglionic stimulation as in Fig. 4 but rate of stimulation 15/sec., (b) without stimulation, (c) during supramaximal stimulation of the preganglionic fibres for 15 sec. once per min.

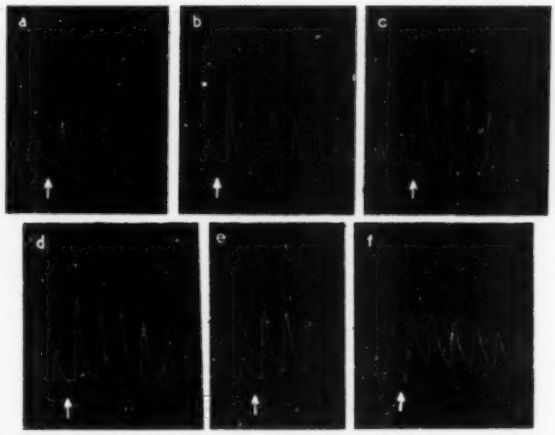

FIG. 6.—Cat under chloralose, 2.1 kg. Normal nictitating membrane. Submaximal preganglionic stimulation for 5 sec. twice per min. Rate of stimulation 2/sec. Intra-arterial injection to the ganglion of 1 μg. 5-HT at arrow. Time interval between records 20 min. The following intravenous injections were made 5 min. before the records: 400 μg. atropine before (b), 500 μg. mepyramine before (c), 1 mg. cocaine before (d), 4 mg. cocaine before (e), 8 mg. cocaine before (f).

depressed by a previous intravenous injection of 400 μg. atropine (Fig. 6b), nor of 500 μg. mepyramine (Fig. 6c), nor of 1 mg. cocaine (Fig. 6d). After 4 mg. cocaine (Fig. 6e), however, and especially after the intravenous injection of 8 mg. cocaine (Fig. 6f), the response to 5-HT was clearly reduced.

DISCUSSION

When 5-hydroxytryptamine (5-HT) was injected intravenously into spinal preparations, observations slightly different from those of Erspamer (1954) were made. While the height of the contractions of the previously denervated nictitating membrane increased with increasing doses of 5-HT, the contractions of the normal membrane increased at a different rate, being comparatively smaller after the injection of 10–30 μg. 5-HT, but exceeding the contractions of the denervated membrane after injections of 100–300 μg. 5-HT.

This change in ratio was not observed in the perfused head. In this preparation 5-HT was always found to be more active on the denervated side; its action was similar to that of adrenaline. Thus 5-HT had a direct action on the smooth muscle of the nictitating membrane, an action which has also been observed by Thompson (1955), who found that 5-HT stimulated the isolated preparation of the nictitating membrane.

Further experiments showed that this direct action of 5-HT on the membrane was potentiated by cocaine, thus providing another example for the view expressed by Fleckenstein and Bass (1953)

that cocaine mimics the effect of previous denervation, causing what they called a " pharmacological denervation " of the nictitating membrane.

The greater response of the normal side (compared with the denervated membrane) after the intravenous injection of larger amounts of 5-HT into spinal preparations may be a finding which is in parallel to the observation by Bülbring and Burn (1938) that " in some cats the response of the denervated membrane to large doses of adrenaline is less than that of the normal membrane." It may, however, also be due to a stimulation of the superior cervical ganglion by higher doses of 5-HT, which would have an additive effect.

This stimulant action on the ganglion was first observed by Robertson (1954a), who injected 1–25 μg. 5-HT into the perfused superior cervical ganglion. In the present experiments 5-HT was injected intra-arterially into the blood supplying the ganglion, and it was found to stimulate about 70% of the preparations. That the contraction of the nictitating membrane was due to an action of 5-HT on the ganglion was shown by comparing the response to injections of 5-HT to the ganglion with the response to injection to the membrane. Furthermore, the action of 5-HT on the ganglion was blocked by substances which did not interfere with its direct action on the membrane.

The stimulant action of 5-HT on the ganglion was similar to that of histamine and pilocarpine, although these last two substances had no direct action on the nictitating membrane (Trendelenburg, 1954, 1955). The action of all three substances on the ganglion was abolished by the depolarizing ganglion-blocking substance nicotine, but was left unchanged by the non-depolarizing substance hexamethonium. Histamine, pilocarpine, and 5-HT were, furthermore, similar in that their ganglionic actions were reduced or abolished by the intravenous injection of cocaine in amounts which did not interfere with ganglionic transmission. However, the effect of 5-HT was less readily abolished by cocaine than that of histamine or pilocarpine.

Finally, 5-HT was similar to histamine and pilocarpine in potentiating transmission through the ganglion. This was observed not only after small amounts of 5-HT which themselves failed to cause ganglionic stimulation, but also after large amounts which stimulated the ganglion cells. There was, however, no nicotine-like paralysis even after the injection of large amounts of 5-HT. Thus it was impossible to confirm Marrazzi's observation that 5-HT blocks transmission (cit. Page and McGubbin, 1953). In spite of the absence of any nicotine-like paralysis, small amounts of 5-HT easily blocked the response to a closely following injection of 5-HT. This observation provides further evidence for the presence of tryptamine receptors in autonomic ganglia, as suggested by Gaddum (1953) and Gaddum and Hameed (1954).

5-HT differed from histamine and pilocarpine only in its not being antagonized by either atropine or mepyramine.

Konzett and Rothlin (1953) recently reviewed the action of various substances on sympathetic ganglia, on the adrenal medulla and on the carotid sinus. They came to the conclusion that for most substances there was a close parallelism in their actions on these three organs. 5-HT provides another example for this parallelism, as it is well known to stimulate the adrenal medulla (Reid and Rand, 1942 ; Lecomte, 1955) and the carotid sinus. Ginzel and Kottegoda (1954) found that the intra-arterial injection of 5-HT close to the carotid sinus caused a fall of blood pressure, which was not prevented by hexamethonium and which was abolished after cutting the sinus nerve. In this connexion it may be of interest to point out that, as Fig. 4 shows, the hypotension after an intra-arterial injection of 5-HT was similar both in magnitude and duration to the potentiation of ganglionic transmission. This observation suggests a certain similarity of the action of 5-HT on sympathetic ganglia and on the receptors of the carotid sinus.

Gaddum and Giarman (1956) have recently shown that the superior cervical ganglion is very rich in 5-hydroxytryptophan-decarboxylase, the enzyme responsible for the synthesis of 5-HT, although the ganglion does not seem to contain any appreciable store of this substance. Nothing is known about a liberation of 5-HT from structures of the ganglion, but it is an interesting fact that 5-HT can be synthesized by a ganglion on whose activity it has been found to exert a facilitating action. The potentiation of ganglionic transmission was observed regularly after the intra-arterial injection of very small amounts of 5-HT (0.01–0.1 μg.). This modifying action on ganglionic transmission seems to be of more importance than the stimulation observed after the injection of larger amounts of 5-HT.

Summary

1. When 5-HT was injected intravenously into the spinal cat the effect on the previously denervated and normal nictitating membranes depended on the dose. Small amounts caused a greater contraction of the denervated membrane, whereas

large amounts caused a greater contraction of the normal membrane.

2. In the perfused head of the cat 5-HT had an adrenaline-like action on the nictitating membrane but was less effective than adrenaline. In this preparation 5-HT caused a larger response on the denervated than on the normal side in all doses.

3. The direct action of 5-HT on the nictitating membrane was not blocked by nicotine; it was potentiated by cocaine.

4. Intra-arterial injection of 1–100 μg. 5-HT into the blood supply of the superior cervical ganglion caused stimulation of the ganglion in 70% of the preparations. This stimulation was abolished by nicotine and by cocaine, but it was not affected by hexamethonium, atropine or mepyramine.

5. Intra-arterial injection of 5-HT into the blood supply of the ganglion potentiated the response of the nictitating membrane to submaximal preganglionic stimulation but not that to supramaximal stimulation. The potentiation of ganglionic transmission was reduced by cocaine, but was unchanged by atropine and mepyramine.

6. The results are taken as evidence for the presence of tryptamine receptors in the superior cervical ganglion.

I wish to express my thanks to Professor J. H. Burn for his advice and guidance throughout this work, and to Mr. H. W. Ling for his very careful assistance with the perfusion experiments. I am indebted to Dr. H. W. Barber, of Messrs. May & Baker Ltd., for a supply of 5-hydroxytryptamine.

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THE RELATIVE EFFECTS OF GANGLION-BLOCKING COMPOUNDS ON THE SYMPATHETIC AND PARASYMPATHETIC GANGLIA SUPPLYING THE CAT HEART

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The use of ganglion-blocking drugs in clinical medicine is limited by the fact that they attack sympathetic and parasympathetic ganglia indiscriminately (Paton and Zaimis, 1952), and this has prompted the search for compounds which might exhibit a selective action on one or other of these autonomic pathways. One method of seeking selective blocking action lies in the study of the transmission process at the two sites to discover if any differences between them suggest a method of differential block. Both sites are, however, cholinergic (Suden, Hart, and Marrazzi, 1952; Perry and Talesnik, 1953). The latter workers showed that the ganglionic action potential of the ciliary ganglion is remarkably similar to that of the superior cervical ganglion save in its shorter duration, which may well be a characteristic not of the parasympathetic system but only of the ciliary ganglion from which neurones run to supply the pupillary muscles. Moreover, the ganglion-blocking compounds studied were similar in action at both sites; so little hope of determining a selectivity of action was offered by this method.

Another method of searching for such a selective action is the screening of large numbers of compounds for this effect on a few chosen ganglia of either system. Great difficulties in interpretation arise, however, because of the differing sensitivity not only of different animal species but of different end-organs within one particular species. Thus, to compare the effect of a drug on the nictitating membrane of the cat (sympathetic ganglion-block) with the effect on the guinea-pig ileum (parasympathetic ganglion-block) is meaningless. Even if the effects of a drug are tested in a single species, such as the cat, by recording comparatively block of nictitating membrane (sympathetic stimulation) and salivary secretion (parasympathetic stimulation), the extreme sensitivity of the latter to all blocking compounds (Paton and Perry, unpublished) makes comparisons very difficult.

We considered that the interpretation of such results might be easier if the effect of ganglion-blocking compounds was studied concurrently on the same end-organ. For this purpose we had to choose an end-organ with a dual innervation, both presynaptic nerve supplies being accessible for stimulation; this paper describes the results we have obtained in one such preparation, namely, the cat heart.

METHODS

Cats were anaesthetized with ethyl chloride and ether followed by intravenous chloralose (80 mg./kg.). Under artificial respiration the chest was opened widely on one side in order to expose the vagus and sympathetic nerves throughout their course to the heart; the pericardium was left intact. Care was taken to ensure that the blood supply to the thoracic sympathetic ganglion was not interrupted. Blood pressure was recorded through a cannula in the femoral artery. Pulse rate and pressure were recorded by transmission through a cannula in the carotid artery to a rubber diaphragm carrying a light balsa-wood lever which wrote on a kymograph.

Presynaptic parasympathetic fibres to the heart were stimulated in the cervical vagus nerve; presynaptic sympathetic fibres were stimulated in the thoracic sympathetic trunk between the 2nd and 3rd thoracic ganglia. A third stimulating electrode was placed on postsynaptic sympathetic fibres in the accelerator nerve. The nerves and vessels on the other side of the chest were left intact. Platinum-wire stimulating electrodes were used for all nerves, which were kept moist and were often covered with liquid paraffin. Square-wave pulses of 0.5 msec. duration, at a frequency of 17/sec., were used for stimulation of each of the nerves. Suitable pulses were of 5–10 volts. The sympathetic and parasympathetic presynaptic nerves were stimulated alternately at

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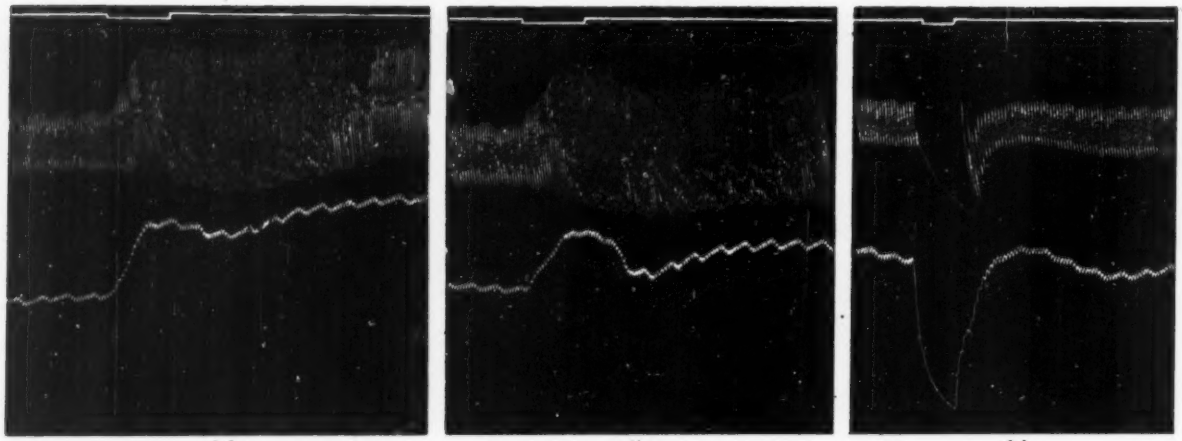

FIG. 1.—Cat, chloralose, artificial respiration. Top trace, periods of stimulation : middle trace, pulse rate and amplitude ; bottom trace, blood pressure. (a) 10 sec. stimulation of sympathetic trunk between 2nd and 3rd thoracic ganglia; (b) 10 sec. stimulation of inferior accelerator nerve; (c) 5 sec. stimulation of cervical vagus nerve.

2 min. intervals for periods of 10 and 5 sec. respectively. Occasional periods of postsynaptic sympathetic stimulation for 10 sec. were interpolated. Recovery from the effects of stimulation for these periods was nearly always complete before the end of the 2 min. cycle. Drugs were administered intravenously into the femoral vein ; hexamethonium and pentamethonium were used as the chlorides, azamethonium as the bromide (" Pendiomide "), and tetraethylammonium (TEA) and tetramethylammonium (TMA) as the bromides.

RESULTS

Effects of Stimulation

Presynaptic Sympathetic Stimulation.—Stimulation of the thoracic sympathetic chain between the 2nd and 3rd thoracic ganglia for 10 or 15 sec. produced an increase of blood pressure and acceleration of the heart rate. The onset of this effect was delayed and began some 3–4 sec. after the start of stimulation. A typical record is shown in Fig. 1(a). The blood pressure rose from 110 to 140 mm. Hg and the heart rate increased from 136 to 168 beats/min. There was also an increase in pulse amplitude. The effect lasted for about 60 sec., heart rate and blood pressure gradually returning to normal. Stimulation was repeated at 2 min. intervals for several hours and the same pattern of response obtained ; the accelerator response was independent of the initial heart rate and blood pressure, but the pressor response was smaller if the initial blood pressure was high. Consequently, we used as the index of the normal response to stimulation the percentage increase in heart rate. This percentage increase was then taken as a 100% response, and responses after blocking drugs were expressed similarly. The initial heart rate was obtained by counting the heart beats during the 5 sec. just before stimulation, and the final rate by counting the beats during 5 sec. at the height of the response. Thus in Fig. 1(a) the initial heart rate was 136 and the final heart rate was 168, so that the percentage increase was $32/136 \times 100 = 24\%$. If after a dose of a ganglion-blocking drug the percentage increase in heart rate was found to be 12%, this is then taken to represent 50% inhibition of transmission.

Postsynaptic Sympathetic Stimulation.—Stimulation of either or both accelerator nerves on the

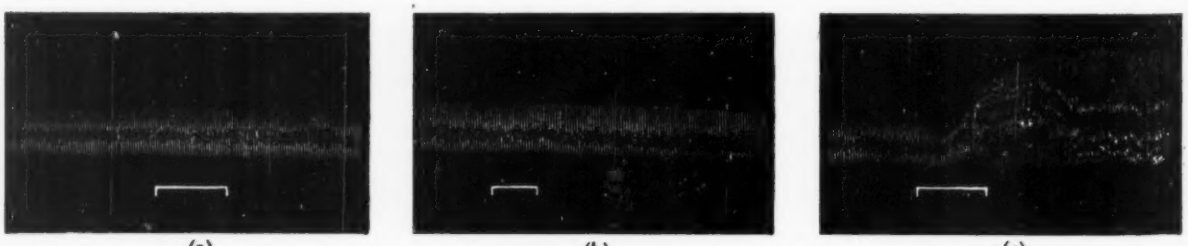

FIG. 2.—Cat, chloralose, artificial respiration. Pulse rate and amplitude after pentamethonium 400 mg. intravenously in divided doses. At signals stimulation as in Fig. 1 of (a) thoracic sympathetic trunk, (b) vagus, (c) accelerator nerve.

one side of the animal for 10 sec. led to the same typical response as that described above after stimulation of the presynaptic sympathetic nerves in the thoracic sympathetic trunk. The magnitude of the effect was similar with either the superior or inferior accelerator nerve, and concurrent stimulation of both did not materially increase the response. A typical record is shown in Fig. 1(b).

The responses were calibrated as described for the response to presynaptic stimulation. The response to postsynaptic sympathetic stimulation was unaffected by the doses of ganglion-blocking drugs used, and test periods of stimulation of this kind were interpolated in all experiments in order to ensure that the effects observed were not peripheral. Unfortunately, it was not possible to ensure that this is true of the parasympathetic innervation ; but the results of Perry and Talesnik (1953) can be used as a basis for inferring that the two systems behave in similar fashion in this respect.

Presynaptic Vagal (Parasympathetic) Stimulation.—Stimulation of the vagus for 5 sec. produced cardiac arrest or very considerable slowing of the heart rate. A typical record is shown in Fig. 1(c).

The time of stimulation used was shorter than that used for the sympathetic nerves, since 10 sec. stimulation of the vagus produced an effect which lasted longer than the interval between successive periods of stimulation (i.e., longer than 2 min.). Longer intervals between successive periods of stimulation made it very difficult to follow the course of block produced by some of the drugs studied. The complete, or almost complete, cardiac arrest lasted throughout the 5 sec. period. Thereafter the heart rate often increased to a level at which it exceeded the normal rate for a short period. The responses were calibrated in the same way as that used for responses to sympathetic stimulation.

Effects of Ganglion-blocking Drugs

"Competitive" Blocking Drugs

We have used in these experiments azamethonium, hexamethonium, pentamethonium, and tetraethylammonium (TEA). All these drugs

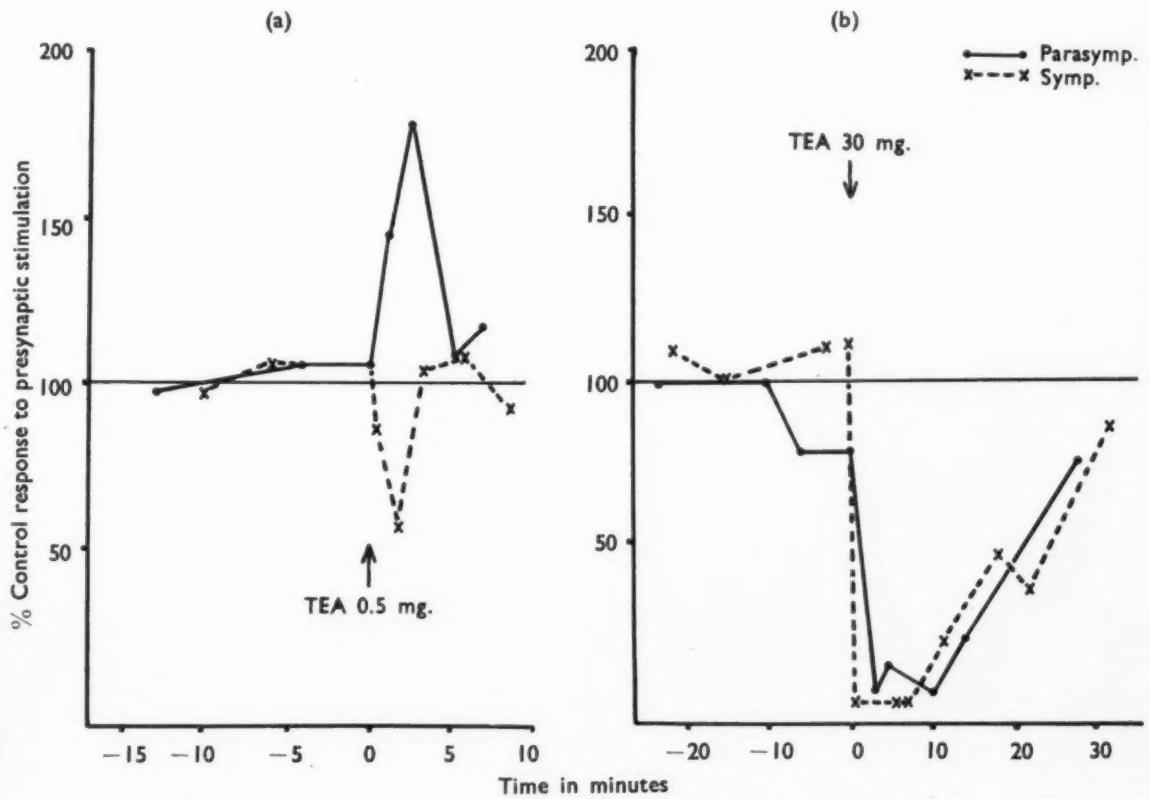

FIG. 3.—Graphs of time-course of block of presynaptic stimulation produced by TEA 0.5 mg. i.v. (a) and 30 mg. i.v. (b). Abscissa, time in min. before and after injection of drug. Ordinate, response of heart as % of control response. Solid line, response to parasympathetic stimulation. Dotted line, response to sympathetic stimulation.

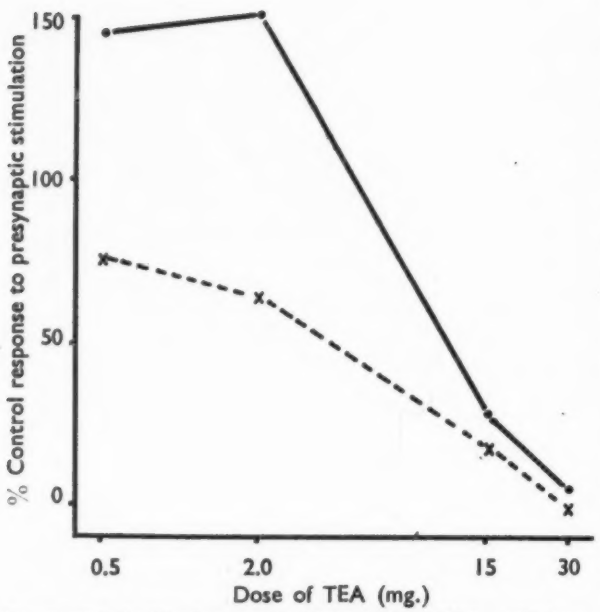

FIG. 4.—Graph of log dose-response lines of effect of TEA. Abscissa, dose of TEA in mg. Ordinate, response of heart as % of control response. Closed circles, response to parasympathetic stimulation. Crosses, response to sympathetic stimulation.

block the response to presynaptic sympathetic stimulation while leaving the effect of postsynaptic (accelerator nerve) sympathetic stimulation intact. Fig. 2 shows, for example, a record obtained using pentamethonium. The block of presynaptic sympathetic responses is always accompanied by a block of the vagal stimulation.

Tetraethylammonium.—The effects of TEA on sympathetic and parasympathetic ganglia appeared to be almost identical, although there was a slight apparent selectivity of action on the sympathetic cells. Fig. 3 shows the effects of graded doses of TEA on each system. Fig. 3(a) illustrates how 0.5 mg. TEA produced a transient block of sympathetic function of about 40%; there appears to have been no such block of vagal function and, in fact, the responses to vagal stimulation were increased, presumably due to the depression of sympathetic tone on the intact non-experimental side, since the two effects ran very parallel. When the dose of TEA was increased to 30 mg. (in the same experiment) there was almost complete block of both systems (Fig. 3(b)); the onset of sympathetic block was, however, rather more rapid, although the duration of block and rate of recovery were similar for both systems. Doses of TEA intermediate between these two doses gave responses intermediate in both magnitude and duration. It will be seen that the block produced by a dose of 30 mg. TEA lasted for some 30 min.

We have attempted to relate the potencies of TEA on the two systems by comparing the dose-response lines for each. The results of one such attempt is shown in Fig. 4. It is obvious that no direct parallelism exists, since the two effects are so closely interrelated that partial block of the sympathetic fibres is accompanied by a potentiation of the effect of vagal stimulation. Nevertheless, at doses which are sufficient to produce block of both, there appears to be no selective action on either system—at high doses the potency ratio being less than 1.5.

In view of the differences in rate of onset, duration and recovery of block, we used another method of illustrating these effects—similar to that used by Paton and Perry (1953). This is shown for another experiment with TEA in Fig. 5. The same type of reaction is seen, namely, a relatively rapid onset of sympathetic block, a slower development of vagal block—the later phases of which are accompanied by partial sympathetic recovery—and, finally, a steady recovery from both effects, reaching normal concurrently.

We have described the effects of TEA in detail, since they provide the basis for describing the action of the other blocking drugs.

Azamethonium.—The results which we obtained with azamethonium are almost identical with those already described for TEA. The onset of paralysis is rapid on the sympathetic ganglion, but the para-

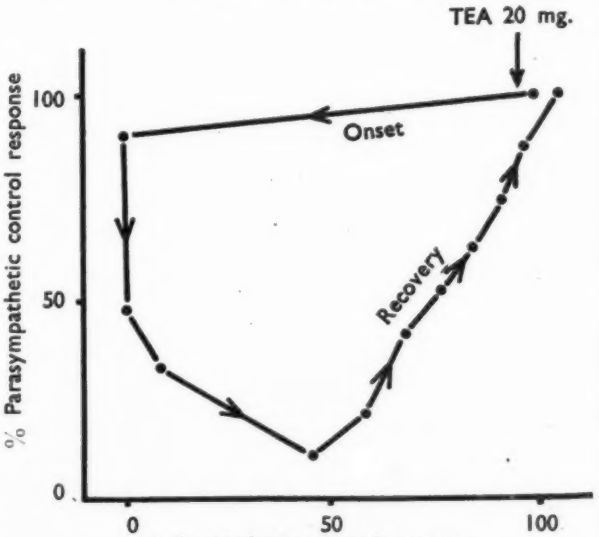

FIG. 5.—Onset-recovery curve for TEA 20 mg. Abscissa, response of heart to parasympathetic stimulation as % of control response. Ordinate, response of heart to sympathetic stimulation as % of control response. Arrows indicate course of block.

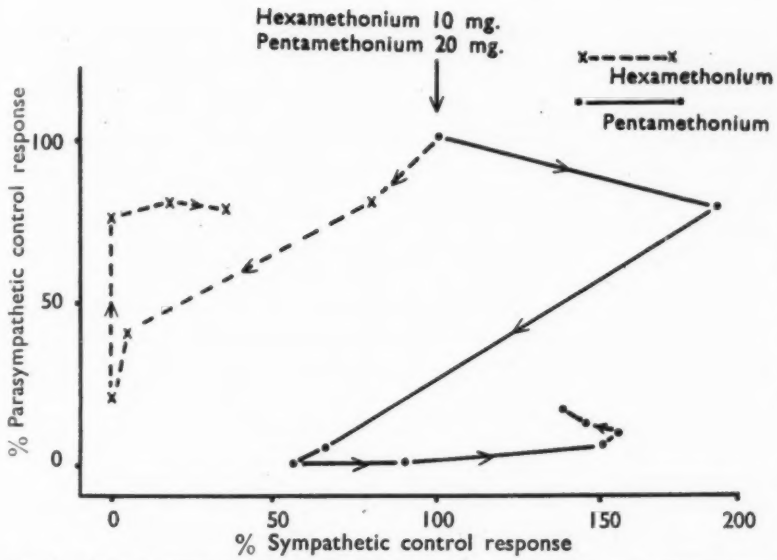

FIG. 6.—Onset-recovery curves for hexamethonium 10 mg. (dotted line) and pentamethonium 20 mg. (solid line). Conventions as in Fig. 5. Arrows indicate course of block.

lysis lasts slightly longer in the parasympathetic ganglia. With 5 mg. the block lasts some 15 min.; with 15 mg. the block is slightly greater, lasting 45 min.

Hexamethonium.—We obtained differing results with hexamethonium. In some experiments the parasympathetic block was slightly more severe; in others the reverse was true. However, the difference was never so great as to suggest a really selective action. The duration of action was also similar in both sympathetic and parasympathetic ganglia. One onset-recovery curve is shown in Fig. 6, where it is compared to a similar curve for pentamethonium. In this particular experiment the parasympathetic block was both less intense and less prolonged than the sympathetic block, although the rates of onset of block were similar.

Pentamethonium.—This compound was the only one with which we observed a consistent selectivity of effect on the cat heart.

Fig. 7(a) shows the dose-response lines obtained in one experiment, and shows that pentamethonium was some 8 times as active in blocking the parasympathetic ganglia as it was in blocking the sympathetic ganglia. In a similar experiment another figure of about 12 was obtained. For comparison Fig. 7(b) shows similar dose-response lines for hexamethonium; in this case there appeared to be a slight selective action (say $\times 3$) on the sympathetic ganglia.

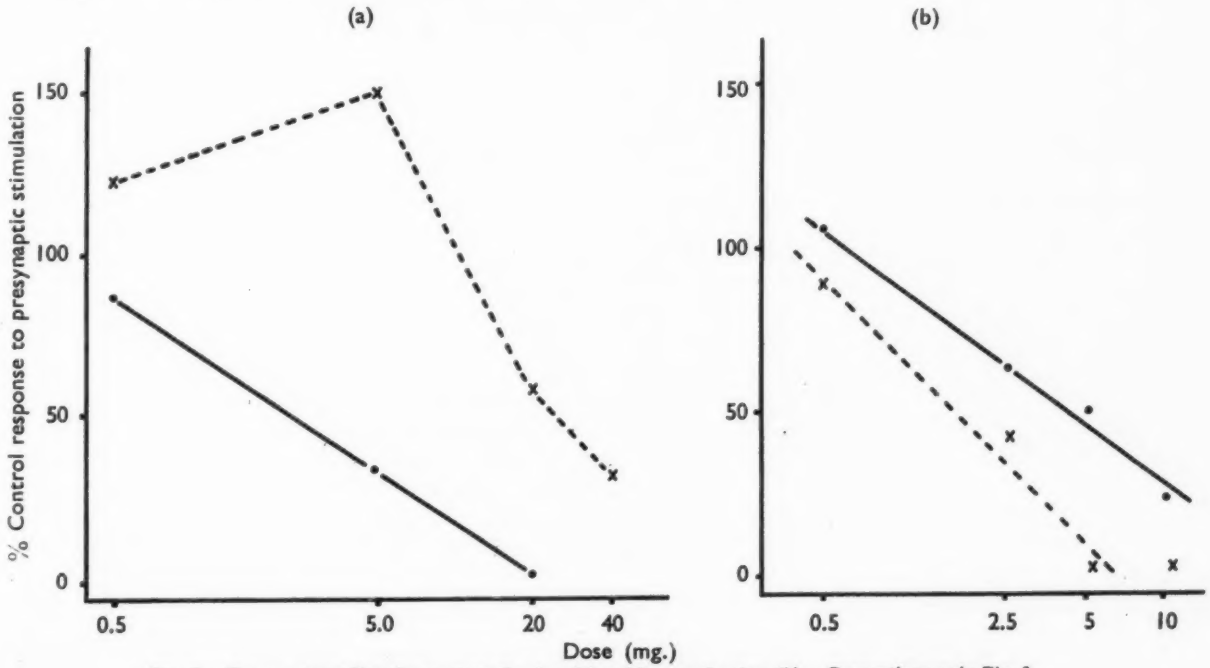

FIG. 7.—Dose-response lines for pentamethonium (a) and hexamethonium (b). Conventions as in Fig. 3.

Moreover, the onset-recovery curves (Fig. 6) make the difference in the effects of these two closely related compounds very obvious indeed. Hexamethonium showed little selectivity either in degree or duration of the block, whereas pentamethonium produced a rapid onset of parasympathetic block accompanied at an early stage by potentiation of the effect of sympathetic stimulation, due presumably to release from parasympathetic tone on the other side of the animal. This was soon followed by partial block of the response to sympathetic stimulation which, however, was of lesser degree than the parasympathetic block. Furthermore, the partial sympathetic block disappeared completely, while the parasympathetic block remained complete. Thereafter there was a further potentiation of the sympathetic response, presumably due, once again, to the temporary absence of parasympathetic tone. Recovery from parasympathetic block was very slow and only the early phase is shown in Fig. 6.

Since in all the experiments quoted only one drug was used, we considered it necessary to confirm that the difference was not one caused by variation in the susceptibility of different cats; consequently, we compared in one experiment the effects of two drugs, and the same differences were observed.

"Depolarizing" Blocking Drugs

Tetramethylammonium.—TMA produced first a marked slowing of the pulse rate and a fall in blood pressure, followed by a prolonged rise of blood pressure and increase of pulse rate above the initial resting levels. The latter effect might be attributed to a stimulation of the sympathetic supply which outlasted the parasympathetic stimulation; or to an effect of adrenaline released by TMA from the adrenals. We repeated this experiment after ligating the blood supply of both adrenal glands, and the TMA then produced only a slowing of the pulse rate, indicating that the later phase of increased pulse rate was in fact due to adrenaline release. Thus it appears that TMA is a more effective stimulant of the parasympathetic than of the sympathetic ganglion cells.

After its initial stimulant effect, TMA blocked the effects of stimulation of the sympathetic and parasympathetic nerves. The degree and time-course of the block of parasympathetic and of sympathetic stimulation were almost identical. This block was relatively short-lived as compared with the block produced by the methonium drugs and closely resembled that produced by TEA.

After a dose of TMA it was necessary to test for block during the phase of increased pulse rate due to release of adrenaline. At this time there appeared to be block of both pre- and postsynaptic sympathetic stimulation. The apparent postsynaptic block was, however, less marked than the presynaptic block, because the pulse rate was already so fast that stimulation failed to produce the usual acceleration. After ligation of the blood supply to both adrenal glands, postsynaptic stimulation was fully effective after large doses of TMA which completely blocked presynaptic stimulation. Furthermore, if adrenaline was administered at this stage, the acceleration produced would again mask the effect of postsynaptic stimulation.

Relative Potencies of Blocking Drugs

We have calculated roughly by graphical methods the relative potencies of the different drugs on parasympathetic and sympathetic ganglia respectively, and these figures are summarized in Table I. Such estimates were only possible from experiments in which a number of different doses were tested.

TABLE I

MEAN RATIOS OF SYMPATHETIC/PARASYMPATHETIC BLOCK, CALCULATED FROM DOSE-RESPONSE LINES AS IN FIGS. 4 AND 7

Drug	No. of Expts.	Ratio
Tetraethylammonium ..	2	0·75
Tetramethylammonium ..	1	1·0
Azamethonium	2	0·75
Hexamethonium	3	0·6
Pentamethonium	2	10·0

DISCUSSION

The method of studying ganglion-blocking drugs which we have described represents, we believe, the first attempt to compare concurrently the effects of such compounds on the ganglion cells of the sympathetic and parasympathetic supply to a single end-organ. Such studies should provide a more useful method of determining a selectivity of action on one or other part of the autonomic system than do the more commonly used methods of testing on more convenient but different end-organs in different species of animals. The relative effects of a drug on the ganglion cells of each system may, of course, depend not only upon inherent differences in sensitivity but also on the accessibility of the cells which are morphologically very differently arranged. Thus administration of the drug by arterial injection close to the end-organ might be expected to produce a selective action on the parasympathetic ganglion cells which are located in the end-organ itself, unlike the

sympathetic ganglion cells which are collected in distinct ganglia and would be proximal to such an arterial injection. We considered that intravenous injection of the drug at a distance from the end-organ provided the best available means of minimizing such possible differences in accessibility, and we have consequently used this technique throughout.

The selection of the heart as the end-organ for study was based on the relative ease of isolation of the presynaptic fibres of both systems supplying it. It is indeed probable that the clinical effects of ganglion-blocking drugs are attributable only to a small extent to their direct action on the autonomic supply to the heart. Further work on other end-organs is most desirable, and the present results merely indicate the possibilities inherent in techniques of this kind.

The results which we have obtained on the rate of onset, duration, and recovery from block of the two systems underline the complexity of the autonomic control. Block of one system may result in potentiation of the effects of the other, possibly due to release of tone ; and this potentiation may well complicate the pattern of any partial block of this second system which may be co-existent with the potentiation. In analysing these effects we have found that the onset-recovery curves, such as those illustrated in Figs. 5 and 6, have been most helpful, and we consider that they go far towards elucidating the pattern of interaction. It is worth pointing out that the potentiation of the sympathetic response seen during parasympathetic block was also observed when the sympathetic stimulation was postsynaptic, indicating that this effect was a peripheral and not a ganglionic one. As might have been expected from previous studies of the transmission process at both sites, most drugs studied showed little or no selectivity of action on the ganglion cells of one or other system. The single striking exception was pentamethonium, which did, apparently, exhibit a marked preferential block of the parasympathetic ganglion cells, being some 10 times more potent in blocking them than in blocking the sympathetic ganglion cells. This was, at first sight, a surprising finding in view of the fact that early work (Paton and Zaimis, 1951) showed pentamethonium to be much more active on the superior cervical ganglion than on the guinea-pig ileum and stressed the possible importance of this apparently selective action. Nevertheless, as Paton and Zaimis (1952) later pointed out, this relatively simple picture failed to survive when it became apparent that different end-organs varied enormously in their sensitivity—as, for example, when Paton and Perry (unpublished) showed that the salivary secretion was blocked first by all the ganglion-blocking drugs tried. The present results indicate that, on the innervation of the heart at least, pentamethonium attacks the parasympathetic cells preferentially and not, as had at first appeared probable, the sympathetic cells. The 10-fold difference in sensitivity between the two systems, which we found with pentamethonium and not with hexamethonium, is not clearly reflected in the clinical activity of the two drugs. The main use of the drugs in clinical practice has been in the treatment of hypertension. What is required in such cases is a selective block of sympathetic ganglia ; and pentamethonium is less effective than hexamethonium. It could be argued that this difference is due to the greater degree of block of parasympathetic ganglia produced by pentamethonium. The situation is obviously complex, and our results suggest that further direct comparisons of the kind we have described will be necessary to clarify it.

Summary

1. A method is described for studying the action of drugs concurrently on the effect of presynaptic stimulation of the sympathetic and parasympathetic ganglia supplying the cat heart. By this method the effects of postsynaptic sympathetic stimulation can also be investigated.

2. A number of ganglion-blocking drugs has been studied using this method. TEA, TMA, hexamethonium, and azamethonium showed no selective blocking action on either the sympathetic or parasympathetic ganglia, in doses which leave the effects of postsynaptic sympathetic stimulation intact.

3. Pentamethonium is 10 times more effective in blocking the parasympathetic ganglia than in blocking the sympathetic ganglia.

4. The time-course of the block produced by these drugs on both systems is described.

5. The use of this and of other similar methods in examining compounds for selective blocking activity on one part of the autonomic system is discussed in relation to the clinical need for such differentiation.

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PRELIMINARY STUDIES ON THE BIOSYNTHESIS OF 5-HYDROXYTRYPTAMINE

BY

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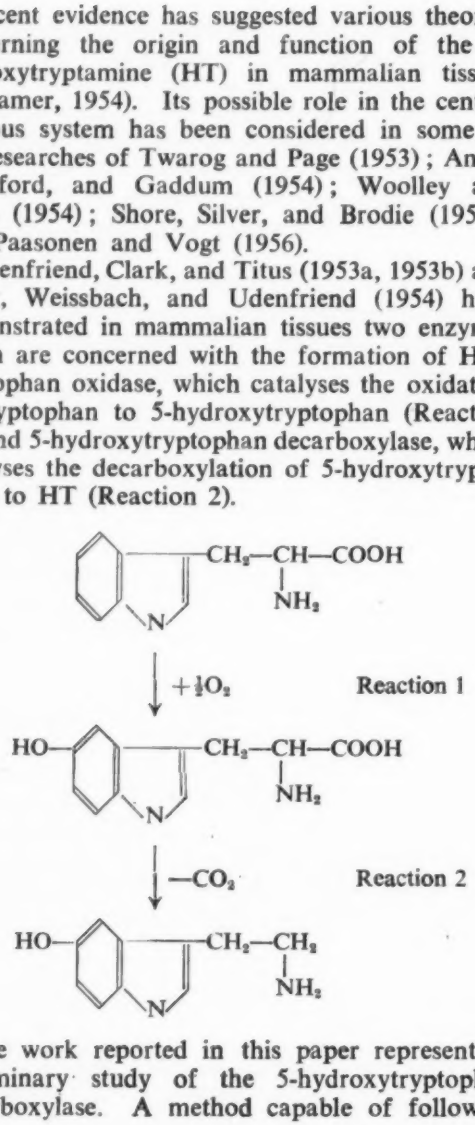
Recent evidence has suggested various theories concerning the origin and function of the 5-hydroxytryptamine (HT) in mammalian tissues (Erspamer, 1954). Its possible role in the central nervous system has been considered in some of the researches of Twarog and Page (1953); Amin, Crawford, and Gaddum (1954); Woolley and Shaw (1954); Shore, Silver, and Brodie (1955); and Paasonen and Vogt (1956).

Udenfriend, Clark, and Titus (1953a, 1953b) and Clark, Weissbach, and Udenfriend (1954) have demonstrated in mammalian tissues two enzymes which are concerned with the formation of HT; tryptophan oxidase, which catalyses the oxidation of tryptophan to 5-hydroxytryptophan (Reaction 1); and 5-hydroxytryptophan decarboxylase, which catalyses the decarboxylation of 5-hydroxytryptophan to HT (Reaction 2).



The work reported in this paper represents a preliminary study of the 5-hydroxytryptophan decarboxylase. A method capable of following the decarboxylation in small amounts of tissue has been developed, and the distribution of the enzyme in the tissues of various species has been traced.

MATERIALS AND METHODS

Preparation of Tissue.—All tissues were freshly excised, carefully cleaned, and weighed in the wet state. Those tissues obtained from the abattoir were frozen with dry ice and carried to the laboratory in a thermos container. The desired amount of tissue was minced finely with scissors and then homogenized in an all-glass homogenizer with M/15 phosphate buffer at *p*H 8.0. The phosphate buffer was prepared after the method of Sørensen by mixing 94.5 ml. M/15-Na_2HPO_4 (9.47 g. anhydrous salt/l.) with 5.5 ml. M/15-KH_2PO_4 (9.08 g./l.).

Aliquots of the homogenates containing the desired quantity of tissue were measured into conical flasks (10 ml.) which served as the reaction vessels. The reaction mixture in these flasks was as follows:

1.	Homogenate of tissue	1 ml.
2.	10 μg. pyridoxal phosphate	0.1 ml.
3.	300 μg. choline *p*-tolyl ether (inhibitor of monoamine oxidase)	0.1 ml.
4.	5-hydroxytryptophan (55 μg. to 440 μg., i.e., final concentration 5×10^{-5}M to 4×10^{-4}M)	0.025–0.2 ml.
5.	M/15-phosphate buffer, *p*H 8.0	3 ml.
6.	Isotonic saline to	5 ml.

The substrate, 5-hydroxytryptophan, was added last, and immediately following its addition the reaction vessels were loosely stoppered, to allow the free interchange of gases, and placed on a shaking device in an incubator maintained at 37° C. In preliminary studies the incubation was allowed to proceed for varying periods of time, but under our standard conditions all incubations were ended after one hour.

The 5-hydroxytryptophan was kindly supplied by the National Drug Company, Philadelphia, Pennsylvania.

Extraction of HT.—This was accomplished by the method of Amin, Crawford, and Gaddum (1954), using

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95% acetone and defatting with petroleum ether. After evaporation to dryness *in vacuo* (at 35° C., external temperature), it was found that the residues could be kept for 48 hr. at −17° C. without loss of activity. Just prior to the assay these residues were dissolved in 0.9% w/v NaCl (1 ml.), and treated with a polyphenol oxidase preparation (Garven, 1956) in order to destroy any catechol amines (such as adrenaline or noradrenaline) in the extracts.

Preparation of Polyphenol Oxidase.—About 20 g. of commercially available mushrooms were put through a household mincer. The solid material was then ground further with silver sand for 15 min. and pressed through linen. Approximately 50 ml. of juice was obtained; this was left overnight in the refrigerator at about 4° C. The juice was then distributed into 0.5 ml. portions in small tubes and freeze-dried. The tubes were sealed and kept in the refrigerator until used. The content of each tube was dissolved in saline (5 ml.) and an appropriate aliquot was used according to potency. In general, 0.2 ml. of a 1:10 dilution was adequate for 0.8 ml. of tissue extract. This preparation destroyed all the catechol amines in extracts of sympathetic ganglia, but not of adrenal medullae.

Bioassay Procedure.—Bioassays were done on the isolated atropinized uterus of the rat in oestrus, according to the method of Amin, Crawford, and Gaddum (1954). Usually a 2 and 2 design was utilized. With certain extracts parallel assays were carried out by M. K. Paasonen, using the hearts of several species of mollusc (Gaddum and Paasonen, 1955). These results were not significantly different from those obtained with the rat's uterus.

These methods proved capable of dealing with quantities of tissue down to 5 mg., although whenever the amount of tissue was of no consideration in this work 250–333 mg. was used.

RESULTS

Action-Time Relationship.—In order to discover the optimal time of incubation, several experiments were done for various periods of time with homogenates of guinea-pig's kidney, which was found to be the richest source of the decarboxylase, and with a relatively high substrate concentration, 8×10^{-4}M. The results of a typical experiment, plotted on a logarithmic scale, are shown in Fig. 1. It is clear that the selection of a time near the asymptote, such as 60 min., is likely to give satisfactory results; solutions were therefore incubated for 60 min. throughout this work.

These results demonstrate another point of some significance. The enzyme is a rapidly functioning one, in that detectable amounts (*ca.* 0.9 μg.) of HT were formed within 20 sec. after the addition of appropriate amounts (in this case 880 μg.) of substrate to homogenates of tissue rich in the enzyme.

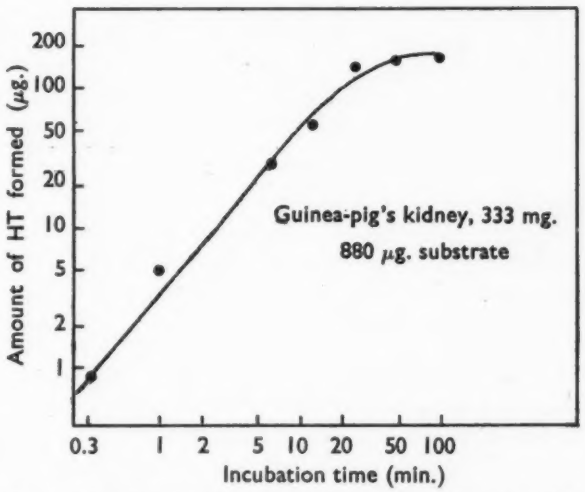

FIG. 1.—The amount of 5-hydroxytryptamine formed by guinea-pig's kidney after incubation for various times. Logarithmic scales. The relation is approximately linear for 45 min. and the reaction then ceased.

Effect of Substrate Concentration.—Fig. 2 shows that the relationship of the enzyme to the concentration of substrate is more complex than its relationship to time. With homogenates of guinea-pig's kidney, enzyme saturation, and probably optimal substrate concentration, were reached at 8×10^{-4} M (880 μg.). However, nervous tissue, such as the stellate ganglia of oxen, behaved in a qualitatively different manner. Optimal substrate con-

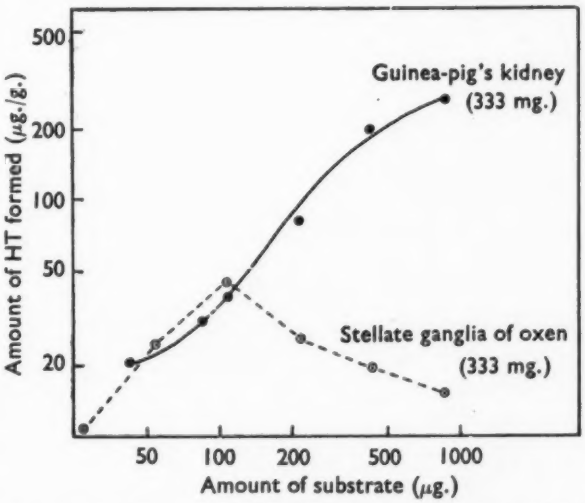

FIG. 2.—The effect of various concentrations of substrate on the amount of 5-hydroxytryptamine formed in one hour. Logarithmic scales. With guinea-pig's kidney 880 μg. of substrate gave a nearly maximal yield. With the stellate ganglia of oxen 110 μg. gave a maximum yield and higher concentrations appeared to inhibit the enzyme.

TABLE I

5-HYDROXYTRYPTAMINE (μG./G. TISSUE) NATURALLY PRESENT AND FORMED FROM ADDED SUBSTRATE (4×10^{-4}M)

No. of observations in parentheses. G and E indicate data of Garven (1956) and Erspamer (1954) respectively

Tissue	Formed				Naturally Present			
	Guinea-pig	Rat	Rabbit	Man	Guinea-pig	Rat	Rabbit	Man
Kidney	200 (9)	120 (3)	45 (3)	—	—	—	0·03 G	—
Liver	25 (3)	20	15	—	—	—	0·4 G	—
Stomach	33 (4)	—	—	—	1·40 E	1·40 E	4·9 E	3·35 E
Spleen	3·3	1·02	4 65	—	1·06 E	2 8 E	19·6 E	0 83 E
Serum	—	—	—	—	0·21 E	0·55 E	3·53 E	0·12 E
Plasma with platelets	0	—	0	0	—	—	—	—
Bone marrow	0	—	0·65	—	—	—	0·33 G	—

centration appeared at a lower level, 10^{-4}M (110 μg.), and substrate concentrations greater than this led to a diminished production of HT.

Tissue Distribution of 5-Hydroxytryptophan Decarboxylase.—A number of tissues from different species of animals have been studied for their decarboxylase activity, an effort having been made to include those tissues which are possible sites of HT production.

Table I lists the data for the non-neural tissues studied, arranged in descending order of decarboxylase activity. For the purpose of comparison, estimates are also given, where possible, of the content of HT in these tissues. It is important to note that these values are not directly comparable with the values for decarboxylase activity, since the magnitude of the latter was determined by the amount of substrate put into the system. It will be observed that, while the HT found in spleen and serum (released from the platelets) was relatively high, the HT-forming capacity of these tissues was of low order or non-existent. Both values were low in bone marrow. A contrary lack of correlation was seen in the kidney and liver, where the ability to produce HT was of a high order, while the HT content was quite low.

The results of experiments on portions of the gastro-intestinal tract of the guinea-pig are summarized in Table II. These figures suggest that

TABLE II

5-HYDROXYTRYPTAMINE (μG./G. TISSUE) NATURALLY PRESENT IN GASTRO-INTESTINAL TISSUES AND FORMED FROM ADDED SUBSTRATE (4×10^{-4}M)

No. of expts. in parentheses

Guinea-pig's Tissue	Formed	Naturally Present (Erspamer)
Stomach, whole	33·0 (4)	1·4
,, fundus	88·8 (4)	—
,, body	50·3 (4)	—
,, pylorus	112·5 (4)	—
Duodenum	127·5	5·0
Ileum	88·4	3·4
Caecum	110·0	—
Colon	15·0	0·7

TABLE III

5-HYDROXYTRYPTAMINE (μG./G. TISSUE) NATURALLY PRESENT IN VARIOUS NEURAL TISSUES AND FORMED FROM ADDED SUBSTRATE (10^{-4}M)

A, data from Amin, Crawford, and Gaddum (1954); P, data from Gaddum and Paasonen (1955); M, parallel assay on heart of mollusc

Tissue	Formed			Naturally Present		
	Dog	Ox	Hog	Dog	Ox	Hog
Sympathetic ganglia*	16·6	44·7 M	—	0 P	0 P	—
Peduncles	—	6·7	—	0 A	—	—
Pons	—	2·6	—	—	—	—
Medulla (whole)	4·7	2·6 M	—	0·03 A	—	—
Caudate nucleus	—	0·6	12·7	0·12 P	0·2 P	0·32P
Hypothalamus	—	0·45	1·72	0·37 P	—	—
Floor 4th ventricle	—	—	1·5	0·1 A	—	—
Cerebellar cortex	0	—	—	0·01 P	—	—
Motor and premotor areas	0	—	—	0·02 A	—	—
Area postrema	—	0 M	0	0·21 A	—	—

* Combined stellate and superior cervical ganglia of dogs; stellate ganglia only of oxen.

there is some correlation between the HT in these tissues and their HT-forming capacity. Both values are at a maximum in the duodenum and seem to decline in regions in either direction therefrom.

Finally, sympathetic ganglia and several areas of the brain were examined for decarboxylase activity. The dissections were done with the aid of M. K. Paasonen. The results, together with values for the normal content of HT in the tissues concerned, are summarized in Table III. These tissues are arranged roughly in order of their decreasing ability to form HT. There is some suggestion of a correlation between the distribution of HT and of the enzyme. The values obtained for both were relatively high in the caudate nucleus and hypothalamus, while in the cerebellar and cerebral cortices the values observed were very low or zero. Truly anomalous situations exist, however, in the sympathetic ganglia and in the area postrema. The former tissue had very high decarboxylase activity, but a very low content of HT (Amin, Crawford, and Gaddum, 1954; and Gaddum and Paasonen, 1955). The area postrema, on

the other hand, presented the opposite picture—a relatively high HT-content (Amin, Crawford, and Gaddum, 1954), but no capacity to make HT.

Discussion

Knowledge of the distribution of an active substance, such as acetylcholine, noradrenaline or HT, in extracts of tissues may give an indication of the part it is likely to play in the body. The discovery of high concentrations of acetylcholine in sympathetic ganglia (Chang and Gaddum, 1933) led to the discovery that it was released on stimulation of preganglionic fibres (Feldberg and Gaddum, 1934). On the other hand, such knowledge may be difficult to interpret. The presence of large amounts of acetylcholine in horse's spleen and human placentae has not as yet been satisfactorily explained.

The amount of an active substance in extracts may depend on a balance between formation and release or destruction, and a knowledge of the rates of these processes is likely to be more valuable than a direct knowledge of the sum of their effects. Active substances may be stored like histamine for long periods in situations where they can have little action because the turnover is small (Ciba Symposium, 1956).

Special difficulties arise with HT, since its presence in a tissue may be due to the accumulation of platelets, which are known to contain large amounts of the material.

The study of the distribution of enzymes concerned with formation is more satisfactory for various reasons: (1) it may give a more direct indication of turnover; (2) it is often applicable to smaller amounts of tissue (Feldberg and Vogt, 1948); (3) it is less likely to be affected by the mode of death of the animal.

The results recorded here are incomplete, but various conclusions may be drawn from them. The differences between the results for different nervous tissues recorded in Table III give strong support to the theory that HT plays a part in the activities of the central nervous system. Any lingering suspicion that the results of studies of the distribution of HT were due to accumulation of platelets can probably now be dismissed. From the available results it is difficult to judge whether the distributions of HT and the decarboxylase in nervous tissue are generally correlated or not, but it is evident that some tissues are exceptional. The area postrema contains particularly large amounts of HT, but no detectable enzyme. Amin, Crawford, and Gaddum (1954) have suggested that HT is probably not produced locally in this area but reaches it from the blood stream. The new results support this suggestion. HT was not found in extracts of sympathetic ganglia even when steps were taken to exclude complications attributable to the presence of adrenaline in the extracts tested (Amin, Crawford, and Gaddum, 1954; Gaddum and Paasonen, 1955; Garven, 1956), but this tissue contained particularly large amounts of the enzyme. This positive result suggests that HT may play a physiological role in this tissue. The nature of this role is obscure, but it may be connected with the sensitizing action of HT observed by Trendelenburg (1956).

The high enzyme activity of extracts of the alimentary canal (Table II) supports Erspamer's (1954) contention that this is an important site of HT production. The lack of enzyme in the platelets and bone marrow is in favour of the view that the HT in the platelets has been absorbed from the plasma (Humphrey and Toh, 1954). It is not in favour of the suggestion of Udenfriend and Weissbach (1954) that HT is synthesized at the site of platelet formation (presumably the bone marrow). The spleen contains large amounts of HT but little enzyme. This is presumably because platelets are broken down in this organ. The distribution of the enzyme through the alimentary canal appears to be similar to that of HT (Table II), maximal concentrations being found in the stomach and duodenum. The fact that comparatively low concentrations of HT were found in the kidneys and livers of rabbits, in spite of the presence of large amounts of the enzyme, may be due to the high activity of monoamine oxidase in these tissues (Blaschko, 1952). The function of the decarboxylase in these organs is particularly puzzling.

Fig. 2 shows that the enzyme in the stellate ganglia of oxen was inhibited by relatively high concentrations of the substrate, while the enzyme in the guinea-pig's kidney was not inhibited in this way. Other experiments, using various amounts of different tissues, seemed to show that this inhibition occurs consistently in nervous tissue and is weak or absent in other tissues. The reasons for this difference are obscure. In the case of cholinesterases a similar phenomenon has been shown to be due to the presence of different enzymes in different tissues (Alles and Hawes, 1940; Mendel and Rudney, 1943). The present observations might also be due to the existence of two enzymes, but there is no other evidence for this at present. Another possible explanation is that in nervous tissue a secondary reaction comes into play, converting the substrate into a derivative which competes with it for the enzyme.

Our method is open to refinement in two respects. The question of the optimal gas phase has not been studied systematically, and it is possible that an anaerobic system might prove more useful and less troublesome, since monoamine oxidase is inactive in the absence of oxygen. Secondly, the use of preparations of the subcellular component in which the decarboxylase resides might also eliminate the problem of enzymic oxidative deamination, because the enzymes responsible may be present in different parts of the cell. These modifications in method are now being studied.

Summary

1. The enzyme 5-hydroxytryptophan decarboxylase, which forms 5-hydroxytryptamine (HT), was studied by incubating tissue homogenates with the substrate and estimating, by bioassay, the HT formed.

2. Kidney, liver and the gastro-intestinal tract showed high activity. Spleen, platelets, and bone marrow showed little or no activity. These results provide evidence against the theories that significant amounts of HT are normally formed in these last three tissues.

3. Sympathetic ganglia, and some parts of the central nervous system, showed activity; this observation supports the theory that HT plays a physiological role in some nervous tissues.

4. The enzyme in nervous tissues was inhibited by an excess of substrate, but in various other tissues such inhibition was not obtained.

5. There are many differences between the distribution of the enzyme and that of the HT which can be extracted from the tissues.

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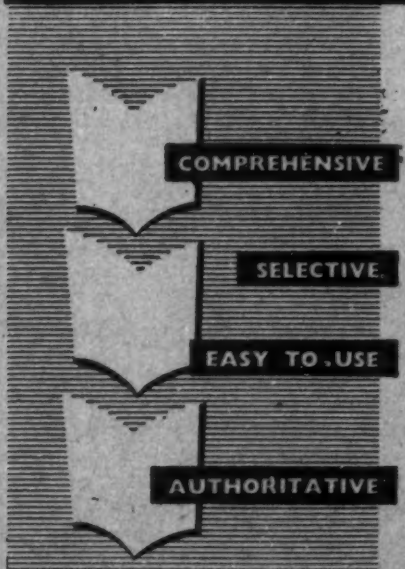

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